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TITLE: THE ROLE OF NEUROPEPTIDE Y (NPY) IN UNCONTROLLED ALCOHOL DRINKING AND RELAPSE BEHAVIOR RESULTING FROM EXPOSURE TO STRESSFUL EVENTS

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14. ABSTRACT

There is high comorbidity between post-traumatic stress disorder (PTSD) and alcohol dependence, indicating that exposure to stressful events increases the risk of alcoholism. Thus, identifying pharmacological targets with potential therapeutic value in treating PTSD-associated alcoholism is critical. During the tenure of this grant, we gained significant insight suggesting that NPY signaling, via the Y1 receptor, plays a protective role against relapse-like ethanol drinking resulting from exposure to stressful events. NPY signaling was also found to modulate the effects of stress on uncontrolled and excessive ethanol drinking. These results have important implications for possible pharmacological medical treatment of alcohol abuse disorders. Specifically, compounds developed as Y1 receptor agonists may represent promising targets to protect against relapse drinking in abstinent alcoholics resulting from exposure to stressful events, including stress stemming from PTSD. Thus, these findings may be considered of high relevance to the U.S. military, as well as the civilian population.

15. SUBJECT TERMS

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INTRODUCTION: People who have been exposed to an extremely traumatic event, such as witnessing a death, receiving a threat of death, or experiencing a serious injury, may develop a set of symptoms known as posttraumatic stress disorder (PTSD). Events that contribute to the development of PTSD are common to individuals placed in a combat environment. Evidence suggests that there is a high comorbidity between PTSD and alcohol dependence. Given the prevalence of PTSD among veterans of war and the increased risk of alcoholism for individuals suffering from PTSD, identifying pharmacological targets with potential therapeutic value in treating PTSD-associated alcoholism may be considered of high relevance to the U.S. military. We proposed neuropeptide Y (NPY) as a possible candidate. NPY is a neurochemical that is present throughout the central nervous system. NPY is involved with a diverse set of biological functions including the integration of emotional behavior such as anxiety and depression. Interestingly, evidence suggests that low NPY levels and deletion of NPY or the NPY Y1 receptor (Y1R) promote high alcohol consumption in mice. Furthermore, combat-related PTSD is associated with decreased plasma levels of NPY, and uncontrolled stress caused by exposure to military survival training results in depletion of plasma NPY levels following extended exposure. Because low NPY levels promote increased alcohol intake, reduced NPY associated with PTSD may be a factor that leaves individuals susceptible to alcoholism. **Therefore, the guiding hypothesis of this proposal was that normal NPY signaling protects against excessive and uncontrolled alcohol drinking and relapse-like drinking caused by exposure to stressful events.** To address this issue, we proposed a set of studies using animal models that assessed the effects of stressors on excessive and uncontrolled drinking and relapse-like drinking. We proposed to use foot-shock as the stressor to elicit increase of ethanol consumption by mice. However, as noted below, we were unable to observe robust reinstatement of ethanol seeking behavior with foot-shock stress, and this stressor also caused *reductions* of ethanol drinking. In line with our alternative approaches in the grant, we then set out to find alternative stressors and approaches with the aim of finding a stressor or stressors that would augment uncontrolled ethanol drinking and relapse-like drinking. We found that a complex stressor (entailing an intraperitoneal injection, and placement into a novel environment with background noise) did significantly increase deprivation-induced relapse-like drinking, and that this effect was more robust in mutant mice lacking the NPY Y1R. Thus, a portion of our data are consistent with the hypothesis that NPY signaling, via the Y1R, is protective against stress-induced relapse-like ethanol drinking. On the other hand, the complex stressor procedure, as well as repeated exposure to intraperitoneal injection, significantly attenuated ethanol consumption using a models of excessive uncontrolled drinking, and mutant mice lacking NPY or the Y1R were more sensitive to the effects of stress. Finally, amygdalar injection of a viral vector that promotes local overexpression of NPY failed to impact the effects of stress on ethanol drinking. Taken together, in addition to implicating roles for NPY and Y1R signaling in the effects of stress on relapse-like ethanol drinking, our data show that the type of stressor, the specific ethanol consumption model, as well as the genetic background of the mouse, all influence how stress impacts ethanol intake in pre-clinical research with rodent models.

BODY (NOTE: Experiments described below are presented in the approximate chronological order in which they were performed to highlight the development of this research program as we gained new information about the stressors and models used. Alternate procedures to better address the research goals of the tasks were implemented when proposed procedures failed, but the questions addressed by each task did not change).

TASK 4: Determine if mutant mice lacking production of NPY show enhanced sensitivity to relapse of alcohol-seeking behavior caused by exposure to foot-shock stress.

Alcoholism is a chronic medical condition involving periods of relapse. Clinical data suggest that exposure to stressful life events is a critical factor contributing to relapse of alcoholism after abstinence. We explored the effect of foot-shock stress on reinstatement of ethanol-seeking behavior

(i.e., pressing a lever that had previously been reinforced with ethanol) after lever responding behavior was extinguished (i.e., lever pressing was not reinforced with ethanol over multiple trials). This procedure is an accepted animal model of relapse and has previously been shown to be useful in studying stress-induced reinstatement of alcohol-seeking in rat models (Weiss and Liu, 2002). Mutant mice lacking NPY (NPY-/-) or normal wild-type mice (NPY+/+) were trained (1-hr sessions) in operant chambers that contained two levers: an active lever reinforced with 14% ethanol and an inactive lever (no reinforcer) to track non-specific responding. After stable ethanol responding, mice were trained under an extinction paradigm where lever pressing did not result in ethanol delivery. Lever-pressing behavior was monitored until no significant differences existed between the ethanol and inactive lever responses for 3 consecutive days. Following the extinction phase, half of the mice were exposed to foot-shocks with an intensity of 0.5 mA (0.5 second duration, a mean 40 second inter-shock interval) for a period of 10 minutes. The remaining mice were not administered shock. Next, mice were placed back in the operant chambers and given access to the ethanol and water levers. Results from this experiment are presented in **Figure 1**. The top panel of Figure 1 (A) shows data collected from mice that received foot-shock stress and the bottom panel (B) shows data from subjects that did not receive foot-shock stress. We ran a three-way repeated-measures analysis of variance (ANOVA) on baseline (BL) data comparing active versus inactive lever pressing for each animal (within subjects factor), NPY-/- and NPY+/+ mice (between subjects factor), and shocked versus no shocked mice (between subjects factor). The main effect of lever (active versus inactive) was the only significant effect [$F(1, 38) = 25.719$, $p < 0.001$] reflecting greater responding to the active (reinforced) versus inactive (no reinforcer) lever. No other effects were statistically significant. A similar repeated-measures ANOVA performed on the data representing the average of the last 3 days of Extinction failed to yield any statistically significant effects. Surprisingly, a repeated-measures ANOVA performed on Reinstatement data also failed to yield any statistically significant effects, though a planned comparison (t-test) indicated that when mice were exposed to foot-shock stress, NPY-/- mice showed significantly more responses to the ethanol lever relative to the inactive lever. However, responding to the active lever did not differ between stressed and non-stressed mice, regardless of the mouse genotype. In general, robust stress-induced reinstatement of responding that has previously reported with rat models (Weiss and Liu, 2002) was not evident. Interestingly, while a Pubmed.gov search reveals over 40 publications that have observed stress-induced reinstatement of ethanol seeking behavior with rat models, there are currently no publications showing stress-induced reinstatement of ethanol-seeking behavior with mice, likely reflecting the difficulties with this procedure in mouse models. Given the complications with this reinstatement model, we immediately sought other methods in an attempt to find better approaches to study stress-induced ethanol seeking behaviors. Deprivation-induced augmentation of ethanol drinking is an accepted animal model of uncontrolled relapse-like drinking (Spanagel, 2000; Spanagel and Holter, 1999), and as we have had success with this approach previously (Sparta et al., 2009) .

Though the foot-shock stressor did not lead to robust reinstatement of ethanol seeking behavior as noted above, we wanted to determine if foot-shock stress might increase ethanol self-administration prior to examining other stressors. We ran a pilot study in which C57BL/6J mice drank 14% ethanol (or water) until a stable consumption baseline was established. Then, ethanol was taken way from half of the mice for one week (EtOH DEP), while the remaining mice continued to have continuous access to ethanol (EtOH CONT). During this time, half of the mice were exposed to foot-shocks as described above. Next, ethanol was returned to the EtOH DEP groups and ethanol consumption measures were recorded over a week period. Data from this pilot experiment are presented in **Figure 2**. We performed a three-way repeated measures ANOVA comparing time of data collection (within subjects factor), stress versus no stress exposure (between subject factor) and ethanol deprivation versus no deprivation (between subjects factor). There was a significant main effect of time of data collection [$F(2, 70) = 27.205$, $p < 0.001$] and a significant three-way interaction between the factors [$F(2, 70) = 3.41$, $p = 0.039$]. A post hoc (t-test) comparison revealed that on the 7^{th} day after stress

exposure, mice in the EtOH DEP and Stress group drank significantly less ethanol than the other groups. These data indicated that exposure to foot-shock stress decreased, rather than increased, ethanol self-administration.

Because foot-shock stress failed to robustly reinstate ethanol seeking behavior and actually reduced ethanol self-administration, we assessed other stressors in accordance with our alternative approaches. Forced-swim is a procedure that has been used to induce stress, and we assessed the effects of forced swim on ethanol consumption in mice. Since previous work has suggested that the effects of stress on ethanol consumption may be more robust in animals with low basal levels of ethanol consumption (Chester et al., 2004; Croft et al., 2005; Little et al., 1999), we also compared the effects of stress on ethanol intake by high ethanol drinking C57BL/6 mice and moderate ethanol drinking BALB/cJ mice. Individually housed mice were given free access to 8% ethanol in one bottle (a lower concentration of ethanol than used above to facilitate drinking in low ethanol preferring BALB/cJ mice) and water in a second bottle with *ad libitum* access to food. Once ethanol drinking stabilized, half the mice were exposed to a forced-swim stressor for 5 minutes, once a day for 5 days, and the remaining mice were only handled (no stress condition). Within each condition (stress or no stress), half the animals were given i.p. injection of a 10 mg/kg dose of CP-154,526 (a corticotropin releasing factor type-1 receptor (CRF1R) antagonist) before each stress or handling trial. The remaining mice were given injection of a vehicle. Results from BALB/cJ mice indicated that exposure to forced-swim stress caused a 4-fold increase in ethanol drinking 2-3 weeks following the stress procedures in the stress/vehicle treated group of mice. However, pre-treatment with the CRF1R antagonist blocked stress-induced increases of ethanol drinking in the stress/CRF1R antagonist treated group. These findings provide direct evidence that the stress-induced increase of ethanol drinking is modulated by the stress-related neuropeptide, CRF. On the other hand, exposure to forced-swim stress did not significantly alter ethanol consumption by C57BL/6 mice. In general, results from this study confirmed that exposure to a stressor (forced swim) can increase ethanol drinking in animals with a moderate level of baseline ethanol consumption, but was not effective at altering ethanol drinking in high ethanol drinking animals. This was problematic as the knockout mice that we proposed to test are maintained on a high ethanol drinking C57BL/6 background. Thus, we sought alterative stressors and approaches. These observations have been published (Lowery et al., 2008a), and the manuscript is appended below (given the direct relevance of this work to the progress of this grant). We also presented an abstract in which we assessed the effects of forced swim stress over multiple inbred strains of mice and found that sensitivity to stress-induced increases of ethanol drinking correlated with baseline level of ethanol intake, where low drinkers were more likely to show stress-induced increases than high drinkers (Lowery et al., 2008b).

TASK 2: Determine if mutant mice lacking production of NPY Y1 receptor show enhanced sensitivity to uncontrolled alcohol self-administration caused by exposure to stress.

Drinking in the dark (DID) procedures have recently been developed to induce excessive and uncontrolled ethanol drinking in C57BL/6J mice which result in blood ethanol concentrations reaching levels that have measurable affects on physiology and/or behavior (Rhodes et al., 2005; Rhodes et al., 2007). This procedure involves giving C57BL/6J mice limited access to 20% ethanol in place of water, beginning 3 hours into the animals dark cycle. Mice were given access to ethanol for 2 hours on days 1-3 of the procedure, and for 4 hours on day 4 (the critical test day). We have used these procedures recently to assess uncontrolled, excessive ethanol intake in C57BL/6J mice (Lowery et al., 2010; Sparta et al., 2008) and used these procedures here to assess the effects of stress exposure on uncontrolled ethanol drinking in mutant mice lacking the NPY Y1 receptor (Y1R-/-) or wildtype Y1R+/+ mice. Repeated intraperitoneal (i.p.) injections of saline have been reported to cause stress-induced increases of ethanol drinking by mice on a high alcohol drinking C57BL/10 background (Little et al., 1999), and thus we used this procedure to induce stress in this experiment.

Data from the test days of this experiment are presented in **Figure 3** (ethanol consumption data are in A and associated blood ethanol concentration data are in B). Mice first experienced a 4 day DID procedure in the absence of stress to assess baseline differences in ethanol drinking between Y1R-/- and Y1R+/+ mice (No Stress). Next, mice again experience the DID procedure and were given an i.p. injection of 0.9% saline (5ml/kg) 30 minutes before ethanol access on each of the 4 days (Stress). Finally, since we noted delayed effects of stress on ethanol consumption as noted above (Lowery et al., 2008a), we retested the mice 3 weeks after stress exposure (Post Stress). We analyzed the test day data using a two-way repeated measure ANOVA comparing the 3 test days (within subjects factor) and genotype differences between Y1R-/- versus Y1R+/+ mice (between subjects factor). Results from consumption data indicated a significant main effect of test day [$F(2, 32) = 4.336$, $p = 0.022$] and a significant main effect of genotype [$F(1, 16) = 5.153$, $p = 0.037$]. Post hoc assessment of ethanol consumption data (t-tests) indicated that Y1R-/- mice drank less ethanol than Y1R+/+ mice after stress exposure and 3 weeks after stress exposure, but did not show significant differences at baseline. While an ANOVA performed on blood ethanol data showed no significant effects, post hoc tests indicated that Y1R-/- mice had lower blood ethanol levels than Y1R+/+ mice on the test day during the week of stress exposure (consistent with lower levels of ethanol intake) but there were no genotype differences at other test points. The high blood ethanol levels at baseline (No Stress) show that mice exhibited excessive ethanol intake, achieving blood ethanol levels of ~100 mg/dl. However, as we noted with foot-shock stress, exposure to i.p. injection stress reduced, rather than increased, ethanol intake and this reduction was still evident up to 3 weeks after stress exposure. Furthermore, stress-induced reductions of ethanol intake were mainly evident in Y1R-/- mice, suggesting that in this case Y1R signaling is protective against stress-induced reductions of ethanol drinking.

TASK 1: Determine if mutant mice lacking production of NPY show enhanced sensitivity to uncontrolled alcohol self-administration caused by exposure to stress.

In a pilot study we had found that exposure to a stressor augmented uncontrolled ethanol drinking to a greater degree in NPY-/- mice (relative to NPY+/+ mice) using DID procedures. Here we performed a larger study, again used DID procedures to model uncontrolled excessive ethanol drinking in NPY-/- and NPY+/+ mice which were maintained on a C57BL/6J background. Since saline injections reduced, rather than increased, ethanol drinking in Y1R-/- mice (above), we used another stress paradigm. As we show below (TASK 5), we discovered that exposure to a complex stress paradigm significantly increased ethanol self-administration in Y1R-/- mice when the stress procedure we administered during periods of ethanol abstinence. This complex stressor consists of given the mouse an i.p. injection followed by relocation to a novel environment with white background noise for 16 hours per day over 5 days just before the initiation of DID procedures. Data from the test day of this experiment are presented in **Figure 4**. Panels A and B show ethanol intake and blood ethanol concentrations (respectively) during testing which began immediately after completion of the stress procedures, and panels C and D show ethanol intake and blood ethanol concentration (respectively) during testing 3 weeks after stress procedures. Data were analyzed with repeated-measures ANOVAs comparing test time (within subjects factor), genotype differences between Y1R-/- and Y1R+/+ mice (between subjects factor), and stressed versus non-stressed (Unstressed) mice that did not receive stress exposure but remained in their homecages during the stressor procedure (between subjects factor). Results from ethanol consumption data revealed main effects of the stressor condition (stress versus no stress) [$F(1, 26) = 4.214$, $p = 0.05$]. Post hoc comparisons (t-tests) showed that during the initial test (panel A), NPY-/- mice exposed to stress drank less ethanol than all other groups. During the test conducted 3 weeks after stress exposure (Panel C), stress-induced reduction of ethanol drinking was evident in both NPY-/- and NPY+/+ mice. A repeated-measures ANOVA performed on blood ethanol concentration data revealed a significant interaction effect between genotype and the stress versus no stress condition [$F(1, 17) = 5.042$, $p = 0.01$], and planned comparisons showed the same group differences noted in consumption data. Thus, similar to the

experiments reviewed in TASK 2, stress exposure reduced, rather than increased, excessive ethanol drinking in mice. Further, NPY-/- mice appear to be more sensitive to stress-induced reductions of ethanol drinking since the effects of stress were evident in NPY-/- mice when tested immediately after the stress exposure while stress-induced reductions of ethanol drinking did not emerge until 3 weeks after stress exposure in the NPY+/+ mice. Thus, again we found evidence that NPY signaling is protective against stress-induced inhibition of excessive ethanol drinking in mice. A manuscript related to experiments from TASK 1 and 2 is in preparation.

TASK 5: Determine if mutant mice lacking production of the NPY Y1 receptor show enhanced sensitivity to relapse alcohol-seeking behavior caused by exposure to stress.

Because we could not observe robust relapse-like behavior (reinstatement) induced by foot-shock stress in TASK 1, we used a complex stressor (describe above) and deprivation-induced drinking, an accepted model of relapse-like drinking with rodents (Spanagel, 2000; Spanagel and Holter, 1999). As noted above, the complex stressor included simultaneous exposure to a change in the mouse environment (a new housing room), exposure to daily intraperitoneal injections, and noise generated by air pumps. We originally discovered this stressor in studies that attempted to created ethanol dependence in mice. Mice were placed in chambers and exposed to air or ethanol vapor. We noted that exposure to this novel environment promoted increases of ethanol intake, regardless of air or ethanol vapor exposure (that is, there were no significant differences between air and ethanol exposed groups), consistent with stress-induced increases of ethanol intake. Y1-/- and Y1+/+ mice were given access to a 15% alcohol solution and water in a second bottle for 2-hours per day. After establishing a baseline, mice were removed from the bottle drinking procedure and experienced the stressor (16-hours per day over a 5-day period). After stress exposure, mice were again given access to ethanol for 2-hours per day over a 5 day test, and then a second 5 day stress exposure was applied, followed by another 5 day ethanol consumption test. Data from this experiment are presented in **Figure 5,** and represent raw consumption data (A) and consumption data converted as change from average baseline intake (B). A repeated-measures ANOVA was performed on the raw consumption data set to compare test day (within subjects factor) and genotype (Y1R-/- and Y1R+/+ mice). The test day main effect [$F(14, 448) = 7.847$, $p < 0.001$] and test day by genotype interaction effect [$F(14, 448) = 3.248$, $p < 0.001$] were both statistically significant. Post hoc comparisons (t-tests) showed that while there were no genotype differences during baseline ethanol consumption, after the first 5 day stress procedure and deprivation from ethanol Y1R-/- mice drank significantly more ethanol than Y1R+/+ mice on days 2-5 of the first limited access session (the timing of stress sessions are indicated by arrows in the figure). The significant increase of ethanol consumption following the period of ethanol deprivation is consistent with elevated relapse-like drinking previously reported (Spanagel and Holter, 1999; Spanagel and Holter, 2000; Spanagel et al., 1996; Sparta et al., 2009). During the second limited access session following the second round of stress exposure and ethanol deprivation, Y1R+/+ mice also exhibited a significant increase of ethanol drinking, thus genotype differences were not evident. A repeated measure ANOVA performed on the change from baseline consumption data revealed significant main effects of test day [$F(9, 288) = 2.38$, $p = 0.013$] and genotype [$F(1, 32) = 11.26$, $p = 0.002$], and a significant test day by genotype interaction effect [$F(9, 288) = 2.278$, $p = 0.018$]. Post hoc comparisons (t-tests) showed that Y1R-/- mice drank significantly more ethanol on days 2-5 of the first limited access session, consistent with the raw data set.

A control experiment was performed to verify that stress exposure contributed to the deprivation-induced increase of relapse-like ethanol drinking (when stress was applied during the deprivation period) noted in the data set of Figure 5. Here, mice were exposed to the same ethanol consumption regimen except that during the 5 day ethanol deprivation period (indicated by arrows in the figure) no stress procedure was applied (**Figure 6**). A repeated-measures ANOVA performed on raw

consumption data (Figure 6A) showed a significant main effect of test day [$F(19, 285) = 61.06$, $p < 0.001$] but no other significant effects. Examination of the data indicated that there were no consistent deprivation-induced increases of ethanol consumption by either genotype, suggesting that exposure to stress during the deprivation period was necessary to observe deprivation-induced increases of ethanol intake above. A repeated-measures ANOVA performed on change from baseline consumption data (Figure 6B) revealed significant main effects of test days [$F(14, 210) = 3.498$, $p < 0.001$] and genotype [$F(1, 15) = 8.799$, $p = 0.01$]. Interestingly, the change from baseline data indicated that ethanol consumption, in the absence of stress application, actually declined over time, and reduced ethanol intake was more robust in the Y1R-/- mice.

One possibility that we needed to rule out was that exposure to the complex stressor promoted a general increase in consumption of reinforcing solutions (such as sucrose), or perhaps an increase in caloric need (since ethanol contains calories). We chose a 1% sucrose solution because we have noted that this concentration of sucrose elicits similar volumes of consumption (0.36 $\pm$ 0.06 ml/2-h) that a 10% ethanol solution generates (0.34 $\pm$ 0.04 ml/2-h). Mice were first given 5-days of access to sucrose for 2-hours per day (baseline) and were then given 5-days of treatment with the complex stressor in the absence of sucrose access (indicated by the first arrow in **Figure 7**). After 5 more days of access to 1% sucrose, mice were given a second 5-day exposure to the complex stressor (indicated by the second arrow). Consumption data in this figure are presented as raw consumption data (Figure 7A) or change from baseline sucrose consumption (Figure 7B). A repeated-measures ANOVA was performed on raw consumption data to assess differences between test days (within subjects factor) and genotype differences (between subjects factor). There were significant main effects of test day [$F(14, 462) = 6.538$, $p < 0.001$] and genotype [$F(1, 33) = 4.658$, $p = 0.038$]. Examination of the data indicated that while Y1R-/- mice drank more sucrose solution over the study, stress exposure/sucrose deprivation did not significantly alter sucrose drinking. Similarly, a repeated-measures ANOVA performed in the change from baseline data revealed a significant main effect of test days [$F(9, 297) = 8.654$, $p < 0.001$], but no genotype effect. Again, there were no reliable increases of sucrose consumption following sucrose deprivation/stress exposure. Together, data from this set of studies show that exposure to the complex stressor augmented deprivation-induced relapse-like drinking of ethanol, an effect that was more robust in mice lacking normal Y1R signaling. Thus, the Y1R appears to play a protective role against the effects of stress on relapse-like drinking. Importantly, the effect of stress on increase deprivation-induced relapse-like drinking is specific to ethanol, and did not generalize to another salient and caloric reinforcer. A manuscript related to these data is currently in preparation and will be submitted in the near future.

TASK 3: Determine if transduction of a NPY viral vector (rAAV-FIB-NPY) into the amygdala of C57BL/6J mice protects against uncontrolled alcohol self-administration caused by exposure to stress.

We used DID procedures described above (to promote excessive uncontrolled ethanol drinking in C57BL/6J mice) in combination with a viral vector that causes production and constitutive secretion of NPY from infected cells. We have found that the high ethanol drinking C57BL/6J inbred strain of mice have significantly lower NPY expression in the amygdala when compared to the ethanol avoiding DBA/2J inbred strain, suggesting that low NPY signaling in the amygdala may contribute to the elevated ethanol intake that is characteristic of C57BL/6J mice (Hayes et al., 2005). We used a recombinant adeno-associated viral (rAAV) vector that promotes production and secretion of NPY in transduced cells. In one rAAV vector, the coding sequence for NPY was preceded by the signal sequence for the laminar protein, fibronectin (rAAV-FIB-NPY), because inclusion of this FIB secretory sequence leads to the secretion of NPY from transduced cells (Foti et al., 2007; Haberman et al., 2003). Additionally, a control vector with the coding sequence for green fluorescent protein (rAAV-GFP) was used. Vectors were infused into the region of the amygdala (1.0 μl/10-min) of male

C57BL/6J mice. Animals rested for two weeks following surgery to allow for adequate gene expression and secretion. Mice were then exposed to a DID procedure in which 20% ethanol was given in place of water beginning 3 hours into the animals dark cycle and remained on the cage for 2 hours. Following this baseline measure, mice were exposed to the complex stress (described above) for 16 hours per day over 5 days. Following complex stressor exposure, mice were tested with the DID procedures over 2 days. The data from this experiment are presented in **Figure 8**. Data were analyzed using a repeated-measures ANOVA comparing test days (within subjects factor) and vector condition (between subjects factor). The only significant effect that emerged was the test days factor [$F(2, 28) = 7.846$, $p = 0.002$]. Post hoc comparisons (paired t-tests) showed that mice drank significantly more ethanol on the second day of testing after the stress exposure. Since we have shown above that in the absence of stress exposure ethanol consumption declines over days in C57BL/6J mice, an effect that has also been reported in the literature (Metten et al., 2011; Wang et al., 2003), these data suggest that exposure to the complex stressor significantly increased excessive ethanol intake in mice. However, as there were no differences between mice treated with the control rAAV-FIB-GFP versus the rAAV-FIB-NPY vectors, overexpression of NPY in the amygdala did not protect against increased ethanol intake. While we did not collected blood ethanol levels in this experiment, on day 2 after stress exposure mice consumed ~3 g/kg/2 hours of ethanol, and we have found that mice drinking this quantity of ethanol in a 2 hour period achieve blood ethanol levels of ~100 mg/dl. These observations suggest that NPY signaling in the amygdala does not modulate excessive uncontrolled ethanol intake in C57BL/6J mice. Alternatively, given that this vector promotes constitutive secretion of NPY from infected cells, it is also possible that compensatory alterations developed, masking the effects of the vector.

TASK 6: Determine if transduction of a NPY viral vector (rAAV-FIB-NPY) into the amygdala of C57BL/6J mice protects against relapse-like alcohol-seeking behavior caused by exposure to stress.

As noted above, because we could not observe robust relapse-like behavior (reinstatement) induced by foot-shock stress in TASK 1, we used a complex stressor (describe above) and deprivation-induced drinking, an accepted model of relapse-like drinking with rodents (Spanagel, 2000; Spanagel and Holter, 1999). We used the recombinant adeno-associated viral (rAAV) vectors described above. Animals rested for two weeks following surgery to allow for adequate gene expression and secretion. Next, a baseline of 2 hours of ethanol consumption (15%) was established (water was available in a second bottle). After establishing a stable baseline, mice experienced the stressor, with each exposure lasting 16-hours per day over a 5-day period. During stress exposure mice were deprived of ethanol. After stress exposure, mice were again given access to ethanol for 2-hours per day over a 5 day test, and then a second 5 day stress exposure was applied, followed by another 5 day ethanol consumption test. Data from this experiment are presented in **Figure 9,** and represent raw consumption data (A) and ethanol preference ratio data, calculated as the proportion of ethanol solution consumed relative to total fluid consumption (ethanol solution + water; B). A repeated measures ANOVA was performed on the raw consumption data to compare test day (within subjects factor) and vector condition (between subjects factor). There was a significant effect of treatment days [$F(14, 196) = 3.681$, $p < 0.001$]. Post hoc comparisons (paired sample t-tests) showed that relative to the average consumption of ethanol at baseline, mice (both rAAV-FIB-GFP and rAAV-FIB-NPY treated mice) consumed significantly less ethanol on days 1, 2, and 5 during the first limited access session after the first stress procedure. A repeated measures ANOVA performed on the ethanol preference ratio data also showed a significant effect of treatment days [$F(14, 196) = 4.414$]. Post hoc test (paired sample t-tests) indicated that relative to the average preference ratio at baseline, mice preferred ethanol more on days 1 and 3 of the first limited access session after the first stress procedure and on days 1 and 2 of the second limited access session after the second stress procedure. We also analyzed the average consumption at each phase of the experiment (**Figure**

10A) and the average consumption of ethanol during the first and second limited access session converted as change from average baseline consumption (**Figure 10B**). A repeated measures ANOVA performed on average consumption data to compare vector condition (between subjects factor) and consumption phase (within subjects factor) revealed a significant effect of consumption phase [$F(2, 28) = 10.681$, $p < 0.001$], and post hoc test (paired t-tests) showed that average ethanol consumption during the second limited access phase (followed two stress exposure procedures) was significantly greater than average consumption during the baseline phase. Similarly, a repeated measures ANOVA performed on the change from average baseline data showed a significant effect of consumption phase [$F(1, 14) = 24.404$, $p < 0.001$], showing that ethanol consumption was significantly greater during the second limited access phase (after two stress procedures) relative to intake during the first limited access phase (after one stress procedure). Since we have shown above that in the absence of stress exposure ethanol consumption declines over days in C57BL/6J mice, an effect reported in the literature (Metten et al., 2011; Wang et al., 2003), these data suggest that exposure to the complex stressor significantly increased deprivation-induced increases of ethanol intake, similar to what we observed in experiments associated with TASK 5. While deprivation/stress induced increase of ethanol consumption was not as robust as was observed in TASK 5, animal differences (C57BL/6J mice purchased from a vendor versus in house breeding colonies), or the fact that vector treated mice experience surgery while the Y1R-/- mice did not, could account for between experiment differences. However, the rAAV-FIB-NPY vector did not impact deprivation/stress-induced increases of ethanol intake. As noted in TASK 3, this may indicate that NPY signaling in the amygdala is not critical for modulating deprivation and stress interactions with respect to effects on ethanol intake, but we cannot rule out the possibility that some physiological compensation occurred in response to the constitutive secretion of NPY by the vector.

TASK 6-Related: Determine if blunting of NPY signaling in the amygdala of BALB/cJ mice protects against stress-induced increases of ethanol drinking.

We performed on additional experiment, related to TASK 6, in an attempt to determine if NPY signaling in the amygdala modulates stress-induced increases of ethanol drinking. As noted above, in our work to identify stressors and stress procedures we discovered that low ethanol drinking BALB/cJ mice showed a delayed increase of ethanol drinking stemming from 5 days of daily exposure to forced swim stress (5 minutes per day), an effect that was blocked by pretreatment with a CRF1R antagonist (Lowery et al., 2008a). Thus, we used forced swim stress and BALB/cJ mice. Before the stress exposure procedure, mice were given bilateral injection of the neurotoxin saporin conjugated to NPY (NPY-SAP) into the amygdala. NPY-SAP selectively and locally kills cells expressing NPY receptors (Bugarith et al., 2005). Since NPY signaling in the amygdala is anxiolytic, were first assessed anxiety-like behaviors in these mice and found that mice treated with NPY-SAP did in fact exhibited significant elevations of anxiety-like behavior. We then examined baseline ethanol drinking in these mice, but found that relative to mice treated with the control blank saporin (B-SAP) mice treated with NPY-SAP did not show significant alterations of ethanol drinking (anxiety-like behavior and baseline ethanol drinking are included in our recent publication (Lyons and Thiele, 2010)). Following baseline consumption, mice were exposed to the 5 day forced swim stress procedure, and ethanol drinking was recorded over 4 additional weeks. Data from this experiment are presented in Figure 11. A repeated-measures ANOVA was performed on this data set to assess average ethanol intake by week (within subjects factor), NPY-SAP or B-SAP treatment (between subjects factor), and stressed versus non-stressed mice (between subjects factor). There was a significant effect of week of testing [$F(5, 260) = 4.056$, $p = 0.001$] but no other effects was significant. Surprisingly, contrary to what we found previously with forced swim stress and ethanol intake in BALB/cJ mice (Lowery et al., 2008a), there was no significant effect of stress on ethanol drinking in the present experiment. Since mice did not experience surgery in our previous report, we speculate that the stressors (physiological and psychological) associated with surgical procedures may have masked the effects of forced swim

stress in the present experiment. Thus, this experiment, as above, failed to provide evidence for a role of NPY signaling in the amygdala in the modulation of stress-induced ethanol drinking.

KEY RESEARCH ACCOMPLISHMENTS:

- Establishing that NPY signaling via the Y1 receptor is protective against stress-induced potentiation of relapse-like drinking in mice.

- Established that the increased sensitivity to stress-induced increases of relapse-like drinking in mutant mice lacking the NPY Y1 receptor is specific to ethanol, and thus stress exposure during a period of sucrose deprivation did not augment sucrose drinking when sucrose water returned to mice. Thus, the effects of stress do not generalize to other salient reinforcers and caloric substance (i.e., sucrose).

- Established that stress exposure reduces ethanol intake using a model of excessive uncontrolled ethanol intake. The effects of stress were long-lasting (observed up to 3 weeks following stress exposure), and NPY signaling, via the Y1R, was actually protective against stress-induced reductions of ethanol drinking.

- Established that NPY signaling in the amygdala may not modulate the effects of stress on uncontrolled excessive ethanol drinking or relapse-like drinking in mice.

- Established that the type of stressor can impact how stress modulates responses to ethanol, as a complex stressor (composed in intraperitoneal injection, change in environment and noise exposure) and forced swim stress increased relapse-like drinking and voluntary ethanol drinking, respectively. On the other hand, the complex stressor blunted uncontrolled excessive ethanol drinking, highlighting the interaction between stressor and the specific model employed (excessive drinking versus relapse-like drinking). Foot-shock stress blunted relapse-like ethanol drinking.

- Established that the genetic background of the mouse tested can impact how stress modulates responses to ethanol, as inbred strains with low basal ethanol intake appear to be more sensitive to ethanol-induced increases of ethanol drinking than in bred strains with high basal levels of ethanol drinking.

- Established that destruction of normal NPY signaling in the central nucleus of the amygdala with the neurotoxin saporin-NPY promotes increased anxiety-like behaviors in mice.

REPORTABLE OUTCOMES:

PERSONNEL SUPPORTED BY THIS GRANT: Note that in all references listed in this section below, Todd Thiele (Thiele, T. E.), George Breese (Breese, G. R.), Darin Knapp (Knapp, D. J.), Thomas McCown (McCown, T. J.), Dennis Sparta (Sparta, D. R.) & Dayna Hayes (Hayes, D. M.) received pay from this grant for research effort. All other personnel listed received salaries from other sources.

PUBLICATONS STEMMING FROM GRANT TASKS:

1. Lyons, A. M. & Thiele, T. E. (2010). Neuropeptide Y conjugated to saporin alters anxiety-like behavior when injected into the central nucleus of the amygdala or basomedial hypothalamus in BALB/cJ mice. *Peptides*, 31, 2193-2199.

2. Lowery, E. G., Sparrow, A. M., Breese, G. R., Knapp, D. J., & Thiele, T. E. (2008). The CRF-1 receptor antagonist, CP-154,526, attenuates stress-induced increases in ethanol consumption by BALB/cJ mice. *Alcoholism: Clinical & Experimental Research,32,* 240-248.

PUBLISHED ABSTRACTS STEMMING FROM GRANT TASKS:

1. Lyons, A. M., Navarro, M., Lowery, E. G., & Thiele, T. E. (2009). The effects of stress and neuropeptide Y (NPY) signaling on binge-like ethanol drinking in C57BL/6J mice. *Alcoholism: Clinical & Experimental Research, 33,* 95A.

2. Thiele , T. E., Lyons, A. M., & Lowery, E. G. (2009). Neuropeptide Y (NPY) Y1 and corticotropin-releasing factor (CRF)-1 receptors modulate stress-induced increases of ethanol intake in mice. *Military Health Research Forum 2009, Symposium S26-2, Poster P20-2.*

3. Sparrow, A. M., Lowery, E. G., & Thiele, T. E. (2008). NPY Y1 receptor knockout mice show increased sensitivity to stress-induced increases of ethanol intake and withdrawal-induced anxiety-like behavior. *Alcoholism: Clinical & Experimental Research, 32,* 33A.

4. Lowery, E. G., Sparrow, A. M. & Thiele, T. E. (2008). The effects of stress on ethanol consumption in Balb/cJ, DBA/2J, and C57BL/6J mice. *Alcoholism: Clinical & Experimental Research, 32,* 32A.

5. Sparrow, A. M., Lowery, E. G., & Thiele, T. E. (2007). Amygdalar neuropeptide Y (NPY) signaling modulates stress-induced reductions of food intake in Balb/cJ mice. *Society for Neuroscience Abstracts, Online.*

6. Lowery, E. G., Sparrow, A. M., Breese, G. R., Knapp, D. J., & Thiele, T. E. (2007). The CRF-1 receptor antagonist, CP-154,526, attenuates stress-induced increases in ethanol consumption in BALB/CJ, but not C57BL/6N, mice. *Society for Neuroscience Abstracts, Online.*

7. Thiele, T. E., Knapp, D. J., Overstreet, D. H., Navarro, M., Breese, G. R., & McCown, T. J. (2007). Amygdalar transduction by a rAAV vector causing constitutive secretion of NPY blocks the alcohol deprivation effect and anxiety-like behavior in Alcohol Preferring P rats. *Society for Neuroscience Abstracts, Online.*

8. Lowery, E. G., Sparrow, A. M., Breese, G. R., Knapp, D. J., & Thiele, T. E. (2007). The CRF-1 receptor antagonist, CP-154,526, attenuates stress-induced increases in ethanol consumption in Balb/cJ, but not C57BL/6N, mice. *Alcoholism: Clinical & Experimental Research, 31,* 208A.

9. Hayes, D. M., & Thiele, T. E. (2007). Assessment of ethanol consumption following site-directed infusion of a neuropeptide Y-saporin neurotoxin in C57BL/6J mice. *Alcoholism: Clinical & Experimental Research, 31,* 87A.

INVITED PRESENTATIONS COVERING WORK RELATED TO THIS GRANT:

1. National Institute on Alcohol Abuse and Alcoholism, Intramural Research Program, Bethesda, Maryland (September, 2009). Talk titled A *Role for CRF & NPY Receptor Signaling in the Modulation of Binge-like Ethanol Drinking by C57BL/6J Mice*.

2. Department of Psychology, University of Illinois at Chicago, Chicago, Illinois (May, 2008). Talk titled *Diverse Roles for Neuropeptide Y in Neurobiological Responses to Ethanol*.

3. Department of Psychology, University of Alaska Anchorage, Anchorage, Alaska (April, 2008). Talk titled *The Role of Central Neuropeptides in Stress-Induced Alcohol Drinking*.

4. Bowles Center for Alcohol Studies, University of North Carolina, Chapel Hill, North Carolina (November, 2007). Talk titled *A Role for Neuropeptide Y (NPY) and Corticotropin Releasing Factor (CRF) in the Modulation of Neurobiological Responses to Ethanol*.

5. Department of Psychology, Clinical Program, University of North Carolina, Chapel Hill, North Carolina (February, 2007). Talk titled *The Role of Neuropeptide Y (NPY) in Ethanol Self-Administration*.

CONCLUSIONS: The present work provides evidence that NPY signaling, via the Y1R, protects against stress-induced increases of relapse-like drinking in mice. Thus, relative to normal mice, mutant mice lacking normal production of the Y1R showed significantly greater relapse-like drinking after ethanol was returned following a period of forced ethanol abstinence and stress exposure. Increased sensitivity to relapse-like drinking by Y1R-/- mice was not evident in the absence of stress exposure, and stress did not augment the consumption of another salient and caloric reinforcer (i.e., sucrose), indicating that the effects of stress were specific to ethanol. On the other hand, stress actually blunted ethanol drinking in a model of uncontrolled excessive ethanol intake, and NPY signaling protected against the inhibitory effects of stress on excessive ethanol intake. We failed to find evidence that NPY signaling in the amygdala modulates stress-induced relapse-like drinking. Negative results to not negate a role for the amygdala, as the tools we employed (e.g., the viral vector which promoted constitutive secretion of NPY) may have not been sensitive enough to reveal a role for the amygdala. The use of more sensitive tools, such as vectors that allow for experimenter-controlled “on and off” activation, may be more useful for assessing the role of NPY signaling in the amygdala. Finally, the present work highlights the sensitivity of this line of research to the type of stressor employed, the genetic background of the mouse used, the specific type of ethanol consumption model utilized, and the interaction between these factors. So what does this all mean? Taken together, observations from the present work suggest that pharmaceutical agonists for the NPY Y1R may be effective in reducing the effects of stress on relapse ethanol drinking in abstinent individuals, but such agonists may not be effective in preventing the effects of stress on uncontrolled excessive ethanol drinking in individuals currently abusing ethanol. The present observations with genetic tools (mutant mice) will need to be replicated with pharmacological tools in future work, such

as administering Y1R agonists during stress exposure in an attempt to block stress-induced increases of relapse-like drinking. Positive pharmacological data would further reinforce the possibility that NPY receptor agonist may be useful for preventing stress-related relapse in humans. Since stressors, such as PTSD, are thought to be robust triggers for relapse in humans, the current findings may be considered of high relevance to the U.S. military, as well as the civilian population.

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SUPPORTING DATA & APPENDICES:

FIGURES

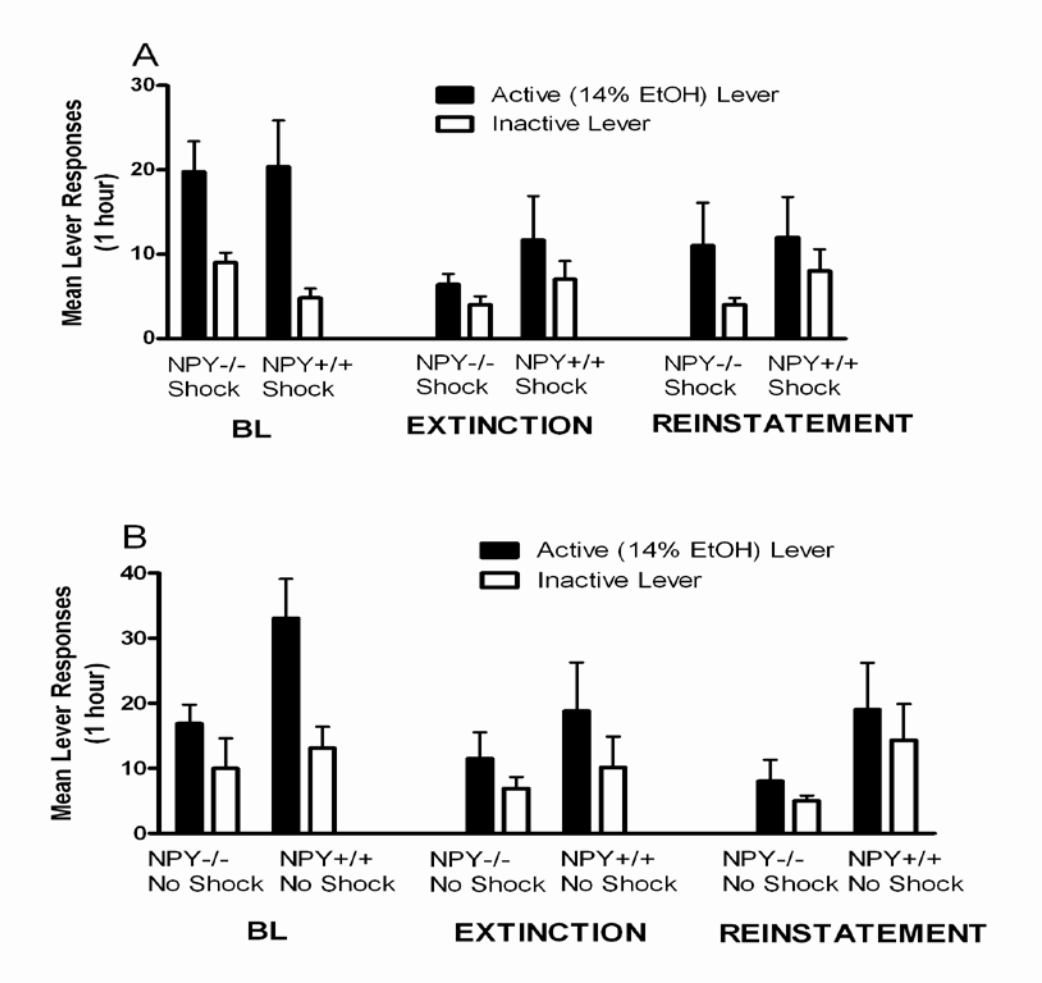

Figure 1: Mean (± SEM) lever response data in NPY-/- (n = 9) and NPY+/+ (n = 12) mice that experienced foot-shock stress (A) or NPY-/- (n = 9) and NPY+/+ mice (n = 12) that had no foot-shock stress (B). Data are presented as average responding during the baseline (BL) phase, average responding during the Extinction phase, and responding in the Reinstatement test day. Solid black bars represent lever presses to the active (14% ethanol reinforced) lever and open bars represent responding to inactive (non-reinforced levers). During the Reinstatement phase, responding to the active lever did not differ between shocked and non-shocked mice, regardless of genotype, indicating that the stressor failed to reinstate responding.

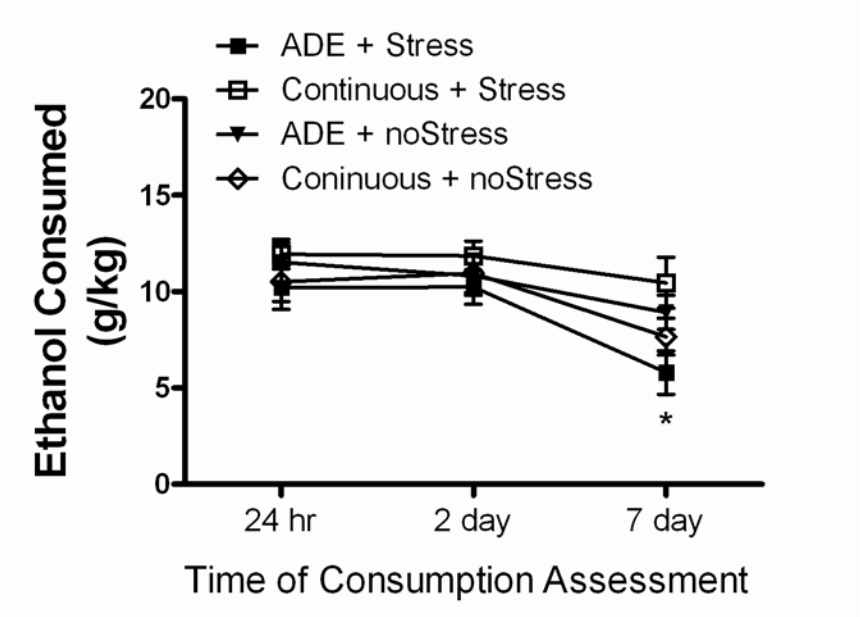

Figure 2: Mean (± SEM) 14% ethanol consumption (g/kg) in mice that had continuous access to ethanol throughout the experiment and received foot-shock stress (EtOH CONT and Stress; n = 9) or no foot-shock stress (EtOH CONT; n = 10) before 7 days of assessment of ethanol intake, or groups of mice that experienced a week of ethanol deprivation and received foot-shock stress (EtOH DEP and Stress; n = 10) or no foot-shock stress (EtOH DEP; n = 10) during the ethanol deprivation period. On data collection over the 7th day, mice in the EtOH DEP + Stress group drank significantly less ethanol than the other groups. * $p < 0.05$.

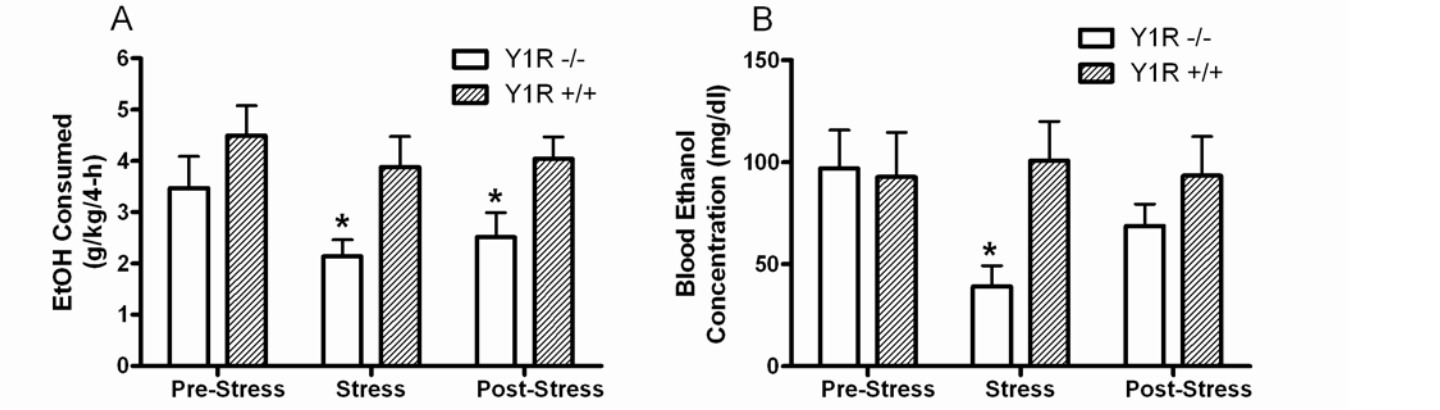

Figure 3: Mean (± SEM) 20% ethanol consumption (g/kg/4 hours; A) and associated blood ethanol concentrations (mg/dl; B) in Y1R-/- mice (n = 8) and Y1R+/+ mice (n = 9) during DID procedures to model excessive uncontrolled ethanol drinking. Data were collected before stress exposure (No Stress), immediately after stress exposure (Stress), and 3 weeks following stress exposure. Relative to Y1R+/+ mice, Y1R-/- mice showed significant reductions of ethanol drinking at both time points after stress exposure. Associated blood ethanol concentrations were also significantly reduced in Y1R-/- mice at the test immediately after stress exposure. * $p < 0.05$ relative to Y1R+/+ mice.

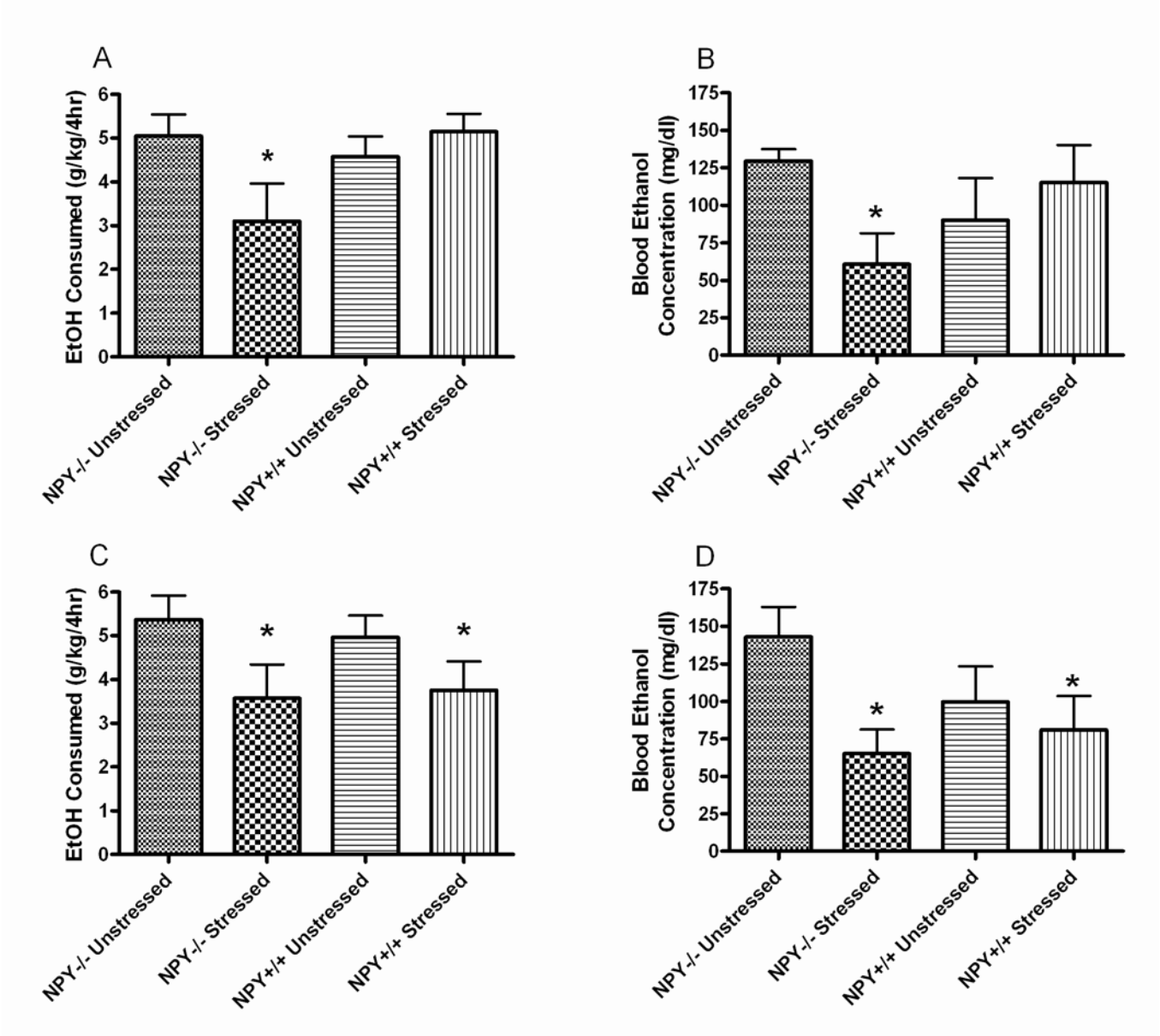

Figure 4: Mean (± SEM) 20% ethanol consumption (g/kg/4 hours; A and C) and associated blood ethanol concentrations (mg/dl; B and D) in Stressed and Unstressed NPY-/- mice (n = 7-8/group) and Stressed and Unstressed NPY+/+ mice (n = 7-9/group) during DID procedures to model excessive uncontrolled ethanol drinking. The top row (A and B) are data collected immediately after stress treatment and the bottom row (C and D) are data collected 3 weeks after stress treatment. Stress treatment significantly blunted ethanol drinking and associated blood ethanol concentration in NPY-/- mice when tested immediately after stress treatment, and stress blunted ethanol drinking and associated blood ethanol levels in both NPY-/- and NPY+/+ mice when tested 3 weeks after stress treatment. * $p < 0.05$ relative to Unstressed groups.

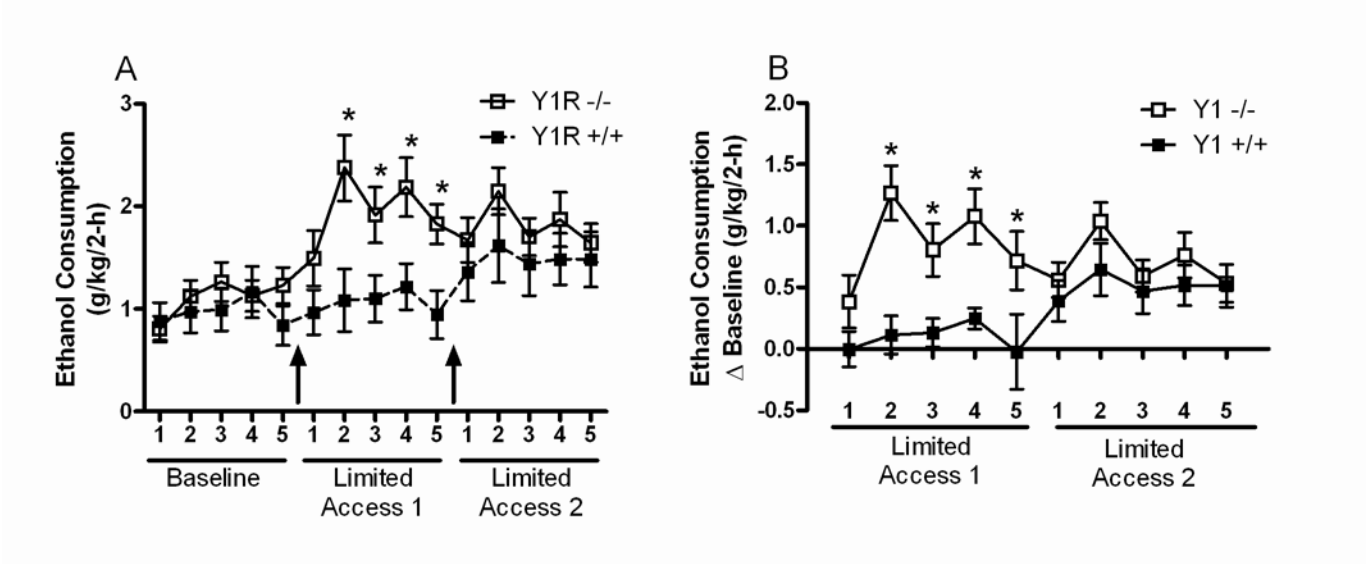

Figure 5: Mean (± SEM) 15% ethanol consumption (g/kg/2 hours; A) and ethanol consumption converted as change from average baseline consumption (B) in Y1R-/- mice (n = 19) and Y1R+/+ mice (n = 17) that experienced stress exposure during periods of ethanol deprivation (arrows indicate the time points of deprivation/stress exposure). Following the first ethanol deprivation/stress exposure, Y1R-/- mice showed elevated ethanol intake relative to Y1R-/- mice. * $p < 0.05$ relative to Y1R+/+ mice.

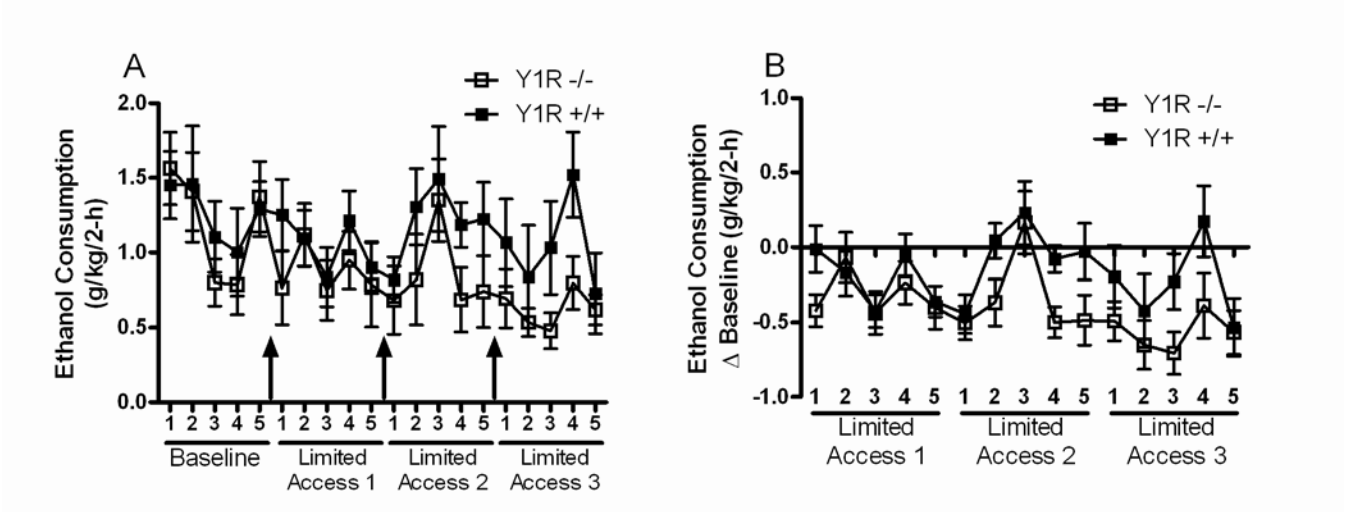

Figure 6: Mean (± SEM) 15% ethanol consumption (g/kg/2 hours; A) and ethanol consumption converted as change from average baseline consumption (B) in Y1R-/- mice (n = 9) and Y1R+/+ mice (n = 8) that experienced ethanol deprivation without stress exposure (arrows indicate the time points of ethanol deprivation). Ethanol deprivation in the absence of stress exposure failed to significantly increase ethanol consumption.

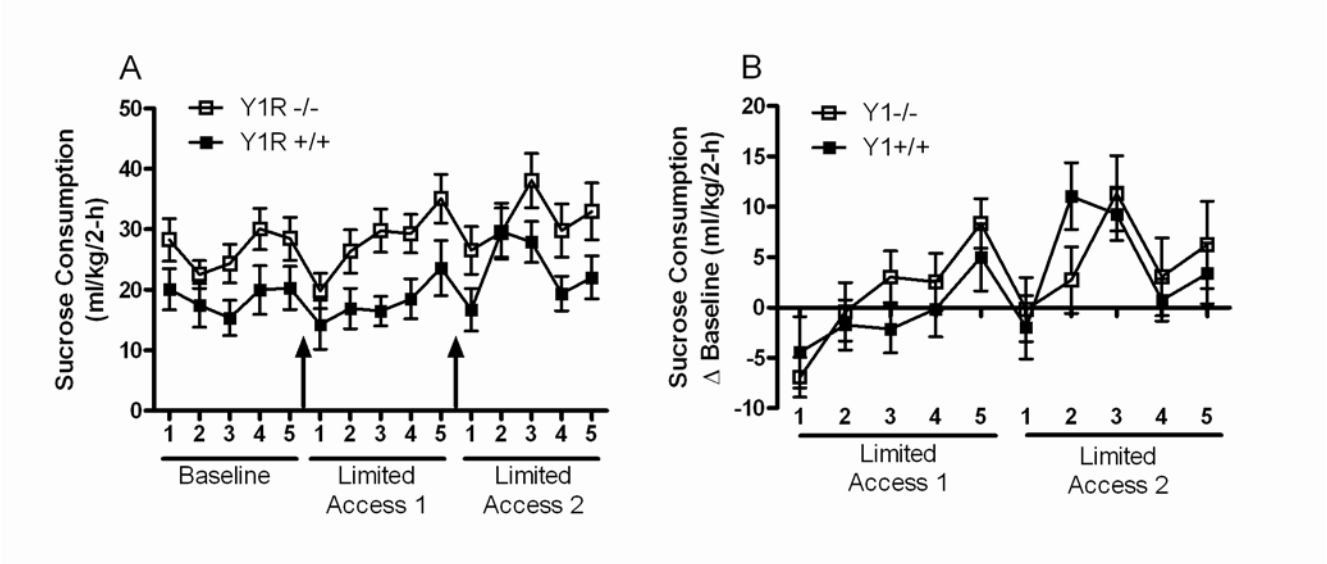

Figure 7: Mean (± SEM) 1% sucrose consumption (g/kg/2 hours; A) and sucrose consumption converted as change from average baseline (B) in Y1R-/- mice (n = 17) and Y1R+/+ mice (n = 18) that experienced stress exposure during periods of sucrose deprivation (arrows indicate the time points of sucrose deprivation/stress exposure). While Y1R-/- mice showed significantly greater overall sucrose consumption over the experiment, sucrose deprivation/stress exposure did not significantly increase sucrose consumption, or differentially impact sucrose consumption between the genotypes.

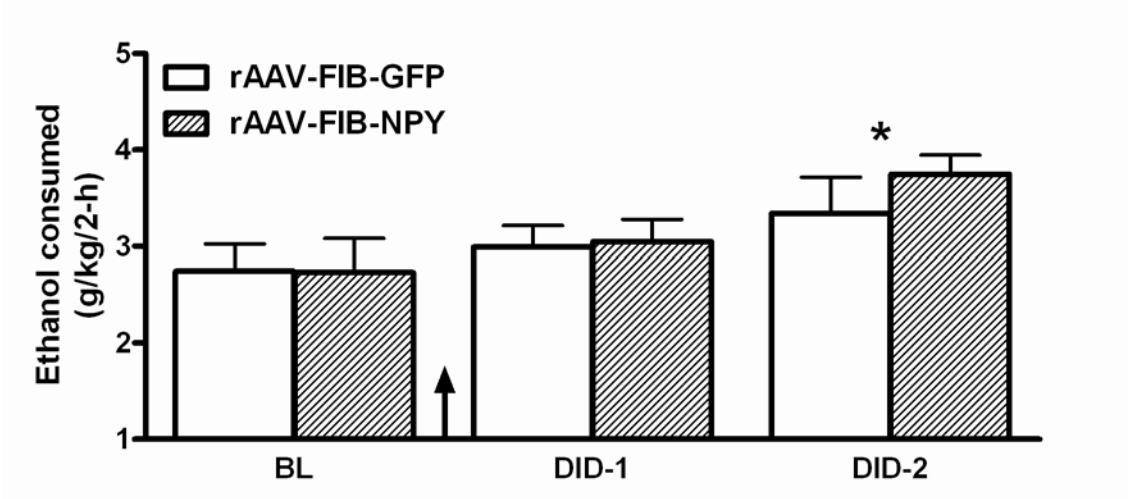

Figure 8: Mean (± SEM) 20% ethanol consumption (g/kg/2 hours) in C57BL/6J mice treated with rAAV-FIB-GFP vector (n = 8) or rAAV-FIB-NPY vector (n = 8) injected into the amygdala. A baseline (BL) of DID ethanol consumption was established, followed by exposure to the stress procedure. Ethanol consumption was significantly increased on the second consumption test after stress exposure (DID-2), but there were no significant differences between the vector treatment conditions. * $p < 0.05$ relative to BL consumption.

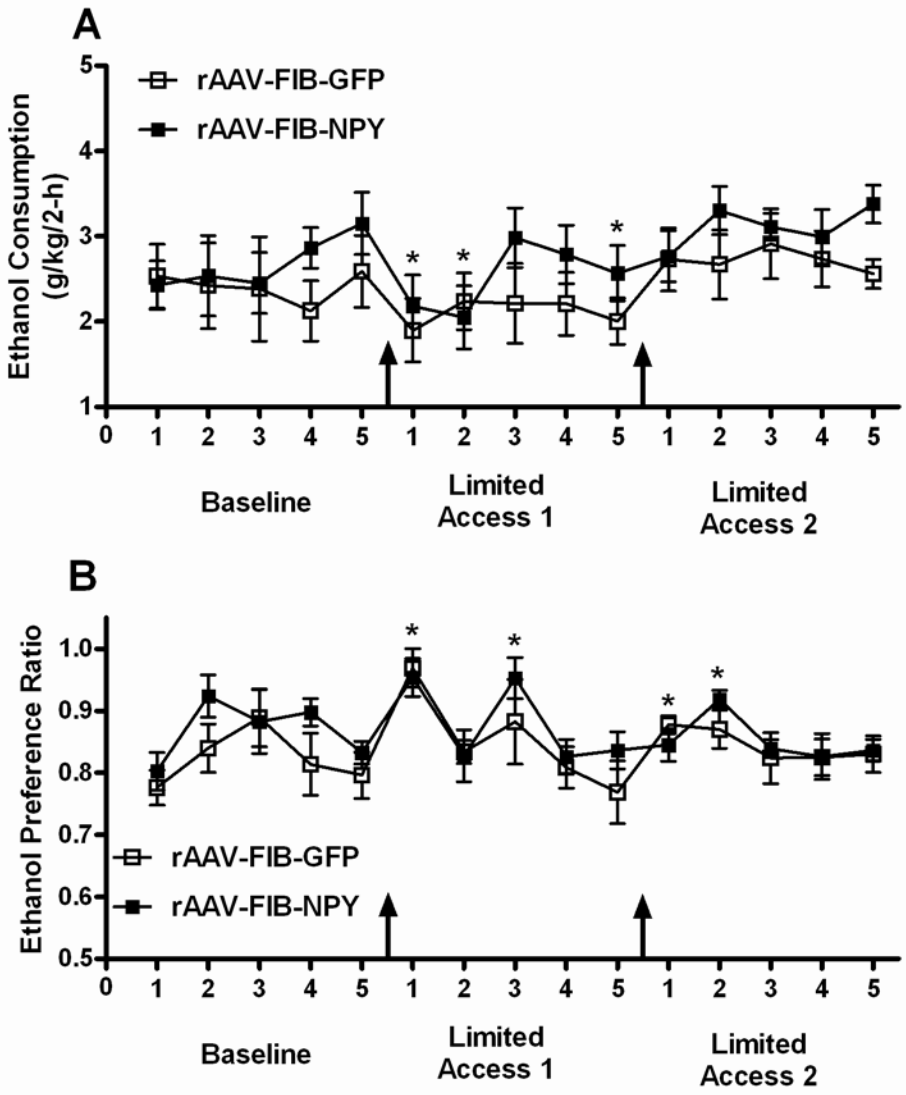

Figure 9: Mean (± SEM) 15% ethanol consumption (g/kg/2 hours; A) and ethanol preference ratios (B) in C57BL/6J mice treated with rAAV-FIB-GFP vector (n = 18) or rAAV-FIB-NPY vector (n = 17) injected into the amygdala. Mice experienced stress exposure during periods of ethanol deprivation (arrows indicate time points of ethanol deprivation/stress exposure). While ethanol consumption was significantly reduced after the first deprivation/stress exposure (A), preference for ethanol was significantly increased after each deprivation/stress exposure (B). * $p < 0.05$ relative to average baseline consumption.

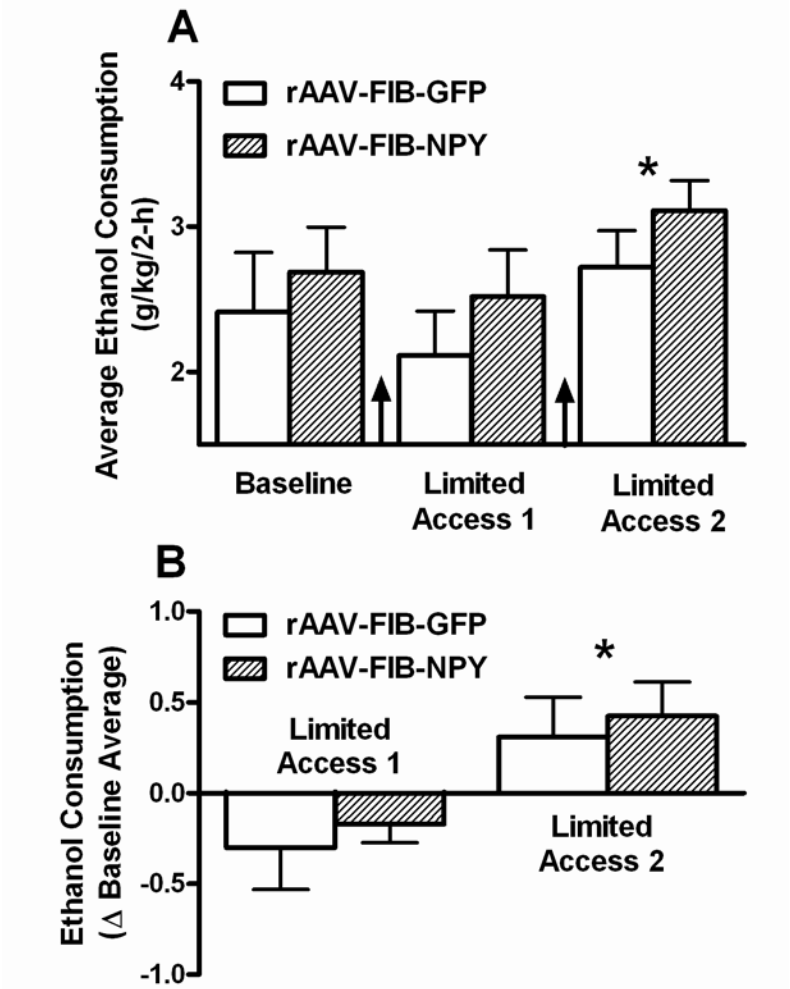

Figure 10: Mean (± SEM) average consumption of ethanol during each phase of the experiment (A) and ethanol consumption during each limited access session converted as change from average baseline consumption. Arrows indicate the time points of ethanol deprivation/stress exposure. While there was no significant effect of vector treatment condition, ethanol consumption was significantly elevated during the second limited access session (relative to baseline consumption), and ethanol consumption was significantly elevated during the second limited access session relative to the first limited access session in data converted as change from average baseline consumption. * $p < 0.05$ relative to baseline (A) or the first limited access session (B).

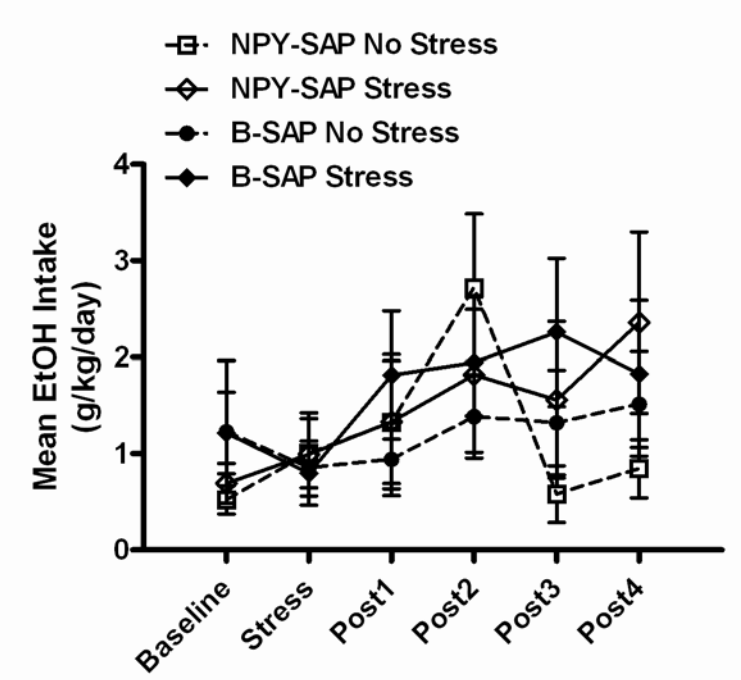

Figure 11: Mean (± SEM) daily average consumption of 8% ethanol by BALB/cJ mice during a week of Baseline intake, the week of stress exposure (5 daily treatments with a 5 minute forced swim stressor), and the four week after the stress procedure (Post1-4). Two weeks prior to the drinking experiment, half the mice were given bilateral treatment into the amygdala of the neurotoxin saporin conjugated to NPY (NPY-SAP No Stress, n = 13; NPY-SAP Stress, n = 14), a toxin the selective kills cells expressing NPY receptor thus blunting local NPY signaling, or were treated with the control black saporin (B-SAP No Stress, n = 15; B-SAP Stress, n = 14). Relative to mice treated with B-SAP, mice treated with NPY-SAP did not show alterations of ethanol consumption, nor did the NPY-SAP interact with the stressor versus no stressor factor.

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Neuropeptide Y conjugated to saporin alters anxiety-like behavior when injected into the central nucleus of the amygdala or basomedial hypothalamus in BALB/cJ mice

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ABSTRACT

Neuropeptide Y (NPY) is a 36-amino-acid neuromodulator that is distributed throughout the central nervous system and has been implicated in a wide range of neurobiological responses including the integration of emotional behavior. The anxiolytic properties of NPY are modulated by NPY signaling in the hippocampus and in the central (CeA) and basolateral (BLA) nuclei of the amygdala. Recently, the neurotoxin saporin, when conjugated to NPY (NPY–SAP), was shown to selectively kill NPY receptor-expressing neurons and has been used as a tool to study the central NPY neurocircuitry involved with feeding behaviors. Here we determined if NPY–SAP can be used as a tool to study the central NPY neurocircuitry that modulates anxiety-like behaviors. BALB/cJ mice were given injection of either NPY–SAP or a control blank saporin (B-SAP) into the CeA or the basomedial hypothalamus (BMH) as a control injection site. The elevated zero maze test was used to assess anxiety-like behavior and NPY–SAP-induced lesions were verified using NPY Y1 receptor (Y1R) immunoreactivity (IR). Results showed that injection of NPY–SAP into the CeA site-specifically blunted Y1R IR in the CeA which was associated with a significant increase in anxiety-like behavior. Injection of NPY–SAP into the BMH, while locally blunting Y1R IR, promoted a compensatory increase of Y1R IR in the BLA and the CA3 region of the hippocampus which was associated with a significant reduction of anxiety-like behavior. The present set of experiments suggest that the NPY–SAP neurotoxin may be a useful tool for studying the NPY neurocircuitry that modulates anxiety-like behaviors.

1. Introduction

Neuropeptide Y (NPY) is a 36-amino-acid neuromodulator that is widely distributed throughout the nervous system [7]. NPY entails anxiolytic properties, first revealed by the observation that central infusion of NPY attenuated anxiety-like behavior in rodents [13]. This was followed by a study demonstrating that site-directed infusion of NPY into the central nucleus of the amygdala (CeA) blunted anxiety-like behavior in rats without altering food intake [12]. NPY signaling in the hypothalamus, on the other hand, modulates feeding behaviors (e.g., [26,28]). More recently, NPY signaling in the basolateral nucleus of the amygdala (BLA) and the hippocampus have been shown to also reduce anxiety-like behaviors [17,23,27,29]. Mutant mice lacking the NPY Y1 receptor (Y1R) show elevated anxiety-like behavior [14], and blockade of Y1R in the amygdala increased anxiety-like behaviors [21], implicating the Y1R in the modulation of anxiety.

In the present experiment, we further explored the role of NPY signaling in the modulation of anxiety-like behavior in BALB/cJ mice, a strain that has been shown to be highly reactive to the effects of stress and exhibits high levels of anxiety-like behavior [2,4]. Our main goal was to determine if the neurotoxin saporin, when conjugated to NPY (NPY–SAP), could be used as a tool to help define the NPY neurocircuitry that modulates anxiety-like behavior. Saporin is a type 1 ribosomal inactivating protein [9] which kills specific populations of neurons by conjugation with proteins that are selectively internalized by the targeted cells [24,30]. NPY–SAP has been shown to selectively kill NPY receptor-expressing neurons (e.g., [3,5]) and has been used as a tool to study the central NPY neurocircuitry involved with feeding and foraging behaviors [3,5,16,22]. The usefulness of NPY–SAP as a tool to study the role of central NPY in anxiety-like behavior has not been established. In Experiment 1, we injected NPY–SAP into the CeA, a region in which NPY injection has been shown to produce anxiolytic effects

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in rodents [12], and subsequently measured behavior on an elevated zero maze, a procedure that is analogous to the elevated plus maze and which is commonly used to assess anxiety-like behavior in rodents [8]. The elevated zero maze has been validated as a test of anxiety from observations that anxiolytic drugs increase open area time in the maze, while anxiogenic drugs reduce open area time [25]. To determine the possibility the treatment with NPY–SAP produced non-specific effects on motor behavior, consummatory behavior and open-field activity were assessed. Lesions were verified by subsequent assessment of Y1R immunoreactivity (IR). As a control for site-specificity, in Experiment 2 a second set of mice were injected with NPY–SAP into the basomedial hypothalamus (BMH), an area in which NPY signaling has been linked to feeding behaviors [3].

2. Methods

2.1. Animals and housing conditions

Male BALB/cJ mice (Jackson Laboratory, Bar Harbor ME) were used in all experiments. Mice were 6–8 weeks old, weighed between 25 and 30 g at the start of the experiments, and were single housed in polypropylene cages with corncob bedding and *ad libitum* access to food and water. Standard rodent chow (Teklad, Madison, WI) and water were available at all times except where noted during experimental procedures. The animal facility was maintained at a temperature of 22 °C with a 12-h/12-h light–dark cycle with lights out at 6:00 p.m. All experimental procedures were approved by the University of North Carolina Animal Care and Use Committee (IACUC) and complied with the NIH Guide for Care and Use of Laboratory Animals (National Research Council, 1996).

2.2. Surgery

Mice were anesthetized with an intraperitoneal (i.p.) injection of a ketamine and xylazine mixture (100 mg/ml and 20 mg/ml, respectively). Using a 33-gauge injection needle, mice received bilateral infusions of NPY–SAP (48 ng/500 nl per side over a 5 min injection) into the CeA (Experiment 1) or BMH (Experiment 2). Injection dose was based on previous work which showed that this dose significantly reduced Y1R IR in the BMH [3]. Control mice were given injection of blank saporin (B-SAP) in the same dose and volume. B-SAP is a control conjugate of saporin with a non-targeted peptide with no known binding site or biological function, and has the same molecular weight as NPY–SAP. NPY–SAP and B-SAP were obtained from Advanced Targeting Systems, San Diego, CA. The stereotaxic coordinates that were used for the CeA were 1.5 mm posterior to bregma, ±2.8 mm lateral to midline, and 4.4 mm ventral to skull surface. The stereotaxic coordinates that were used for the BMH were 1.5 mm posterior to bregma, ±0.4 mm lateral to midline, and 5.5 mm ventral to skull surface. Mice were given 10 days of recovery and to allow time for the saporin to induce lesions.

2.3. Elevated zero maze testing

Ten days after surgery, mice were transported from their vivarium to a room immediately adjacent to the testing room and allowed to habituate for at least 30 min before testing began. The elevated zero maze (Hamilton-Kinder, Poway, CA) was positioned in the center of a room below a ceiling-mounted lamp fitted with a single 25-W red light bulb which provided the only light for the room. Each mouse was individually removed from its home cage and immediately placed just inside a closed area of the elevated zero maze with its nose pointing into the closed area section. The 5 min test session was video recorded with a tripod-mounted camcorder to eliminate the need for an investigator's presence in the testing room. Sessions were scored by treatment-blind investigators for time spent in open or closed areas (s), and the number of open and closed area entries. An animal was considered to have entered the open area if all four paws had left the closed area. Open area time was considered terminated once all 4 paws were placed back into the closed area.

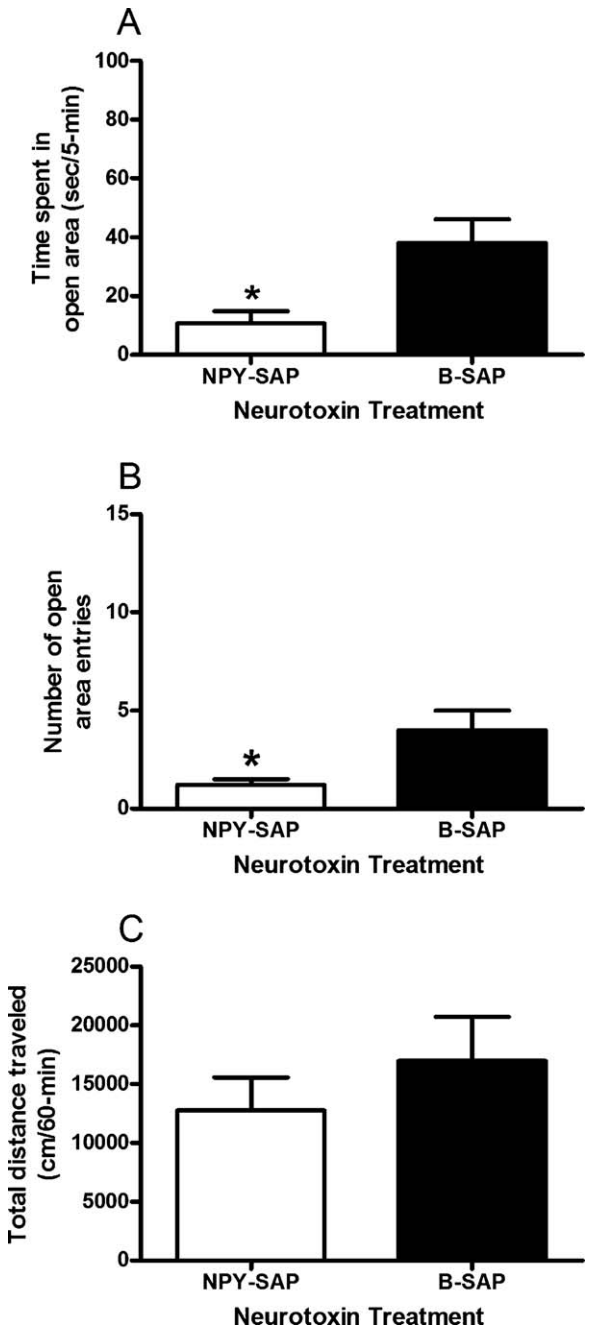

Fig. 1. Results from elevated zero maze and open-field testing in BALB/cJ mice given NPY–SAP or B-SAP injection into the CeA. Relative to B-SAP treated mice, time spent in the open area (A) and number of open area entries (B) were significantly reduced in mice treated with NPY–SAP. There were no significant differences between the NPY–SAP and B-SAP groups in open-field locomotor activity (C). $^{*}p < 0.05$.

2.4. Assessment of consummatory behavior and open-field testing

To determine the potential effects of NPY–SAP treatment on general motor activity, consummatory behavior (food, water, and 10% (v/v) ethanol intake) and open-field locomotor activity were analyzed in a subset of mice that were used in the elevated zero maze test (the remaining mice were used in procedures that involved stress exposure subsequent to elevated zero maze testing and thus were not included in the present analysis). Consummatory measures were collected over 6 weeks. This was followed by open-field testing in which mice were transported to the testing room and allowed to habituate for at least 30 min. Mice were placed into the center of an open-field arena that automatically recorded activity via photo beam breaks (Harvard Apparatus, Inc., Holliston, MA). The open field arena measured 40.64 cm × 40.64 cm × 30.48 cm and was made of clear Plexiglas. Testing sessions were 60 min in duration and the chambers were cleaned with isopropyl ethanol wipes after each session. Total distance traveled (cm) was measured over the course of the session.

2.5. Immunohistochemistry (IHC) procedures

Upon completion of the study (approximately 2 months after receiving infusion of NPY–SAP or B-SAP), mice received an i.p. injection of a ketamine/xylazine mixture (100 mg/ml and 20 mg/ml, respectively) and were then perfused within 10 min transcardially with 0.1 mM phosphate-buffered saline (PBS; pH 7.4) followed by 4% paraformaldehyde in phosphate buffer. Mice were perfused in pairs and counterbalanced by group. Brains were collected and post-fixed in paraformaldehyde for 48 h at 4 °C, at which point they were transferred to PBS. Using a vibrotome, mouse brains were sliced into 40 μm sections and stored in PBS until IHC procedures. The sections were then rinsed in PBS 3 times (10 min each). Sections were blocked in 10% goat serum and 0.1% Triton-X-100 in PBS for 1 h. Sections were then transferred to fresh PBS containing primary rabbit Y1 receptor antibody (1:25,000) for 72 h at 4 °C. Antibody 96106 raised against NPY Y1R was provided by CURE/Digestive Disease Research Center, Antibody/RIA Core, NIH Grant #DK41301 (Los Angeles, CA). After primary incubation, brain sections were rinsed 3 times with PBS and processed with Vectastain Elite Kits (Vector Labs, Burlingame, CA) as per manufacturer's instructions. Sections were visualized by a reaction with 3,3′-diamino-benzidine (DAB, Polysciences Inc, Warrington, PA) in a reaction solution containing 0.05% DAB, 0.005% cobalt, 0.007% nickel ammonium sulfate, and 0.006% hydrogen peroxide. Sections were mounted on glass slides, air-dried, and cover slipped.

Digital images of Y1R IR were taken in candidate brain regions using a Nikon E400 microscope with a Nikon Digital Sight DS-U1 digital camera run with Nikon provided software. For analysis, great care was taken to match sections through the same region of brain and the same level using anatomical landmarks with the aid of a mouse stereotaxic atlas [10]. Densitometric procedures were used to assess protein levels. Flat-field corrected digital pictures (8-bit grayscale) were taken and density of staining was analyzed using Image J software (Image J, National Institute of Health, Bethesda, MD) by calculating the percent of the total area examined that showed signal (cell bodies and processes) relative to a subthreshold background. The size of the areas that were analyzed was the same between animals and groups. The subthreshold level for the images was set in such a way that any area without an experimenter defined level of staining (determined by terminal- and/or soma-positive regions) was given a value of zero. Within each region, the same subthreshold level was used for each slice that was scored. Data from each brain region in an animal were calculated by taking the average counts from the left and right sides of the brain at the specific brain region of interest. For each brain region, photographs were taken at approximately the medial area of the structure (with respect to the rostral–caudal axis). In all cases, quantification of immunohistochemistry data was conducted by an experimenter that was blinded to group identity. For some brain regions, representative slices were not available for 1–2 mice, which is reflected in the degrees of freedom in analysis described below.

2.6. Data analysis

All data is presented as mean ± SEM. For elevated zero maze and open-field locomotor activity data, differences between groups were analyzed using independent-sample *t*-tests. Consummatory data were analyzed using 2 × 6 (saporin treatment × average daily consumption blocked by week) repeated-measures analysis of variance (ANOVA). In all cases, $p < 0.05$ (two-tailed) was used to indicate statistical significance.

3. Results

3.1. Experiment 1: CeA infusion of NPY–SAP

3.1.1. CeA infusion of NPY–SAP is associated with increased anxiety-like behavior

Results from the elevated zero maze test and open-field locomotor activity test in mice treated with CeA infusion of NPY–SAP or B-SAP are presented in Fig. 1. Mice treated with NPY–SAP ($n = 23$) exhibited increased anxiety-like behavior relative to mice treated with B-SAP ($n = 27$), evidenced by a significant reduction of time spent in the open area of the elevated zero maze (Fig. 1A) and a significant reduction in open area entries (Fig. 1B). *t*-Tests performed on open area time [$t(48) = 2.9$, $p = 0.006$] and open area entries [$t(48) = 2.366$, $p = 0.022$] data were both statistically significant, confirming the above conclusions. On the other hand, mice treated with NPY–SAP ($n = 12$) failed to show alterations in locomotor activity relative to mice treated with B-SAP ($n = 15$), suggesting that alterations of elevated zero maze behavior in NPY–SAP-treated mice were not likely related to overall alterations of motor behavior (Fig. 1C). A *t*-test performed on locomotor activity data failed to achieve statistical significance [$t(25) = 0.855$, $p = 0.400$]. Consummatory measures provide further evidence that the NPY–SAP treatment did not impact motor behavior or overall health of the mice as there were no significant differences between NPY–SAP ($n = 12$) and B-SAP ($n = 15$) groups in terms of average food intake (305.47 ± 15.41 g/kg/day versus 299.47 ± 13.78 g/kg/day, respectively), water drinking (196.58 ± 10.33 ml/kg/day versus 215.64 ± 9.24 ml/kg/day, respectively), or ethanol intake (1.14 ± 0.32 g/kg/day versus 1.21 ± 0.28 g/kg/day, respectively). Repeated-measures ANOVAs performed on food and water intake data revealed significant main effects of week [$F(5, 125) = 24.728$, $p = 0.001$; $F(5, 25) = 4.456$, $p = 0.001$, respectively], but no other effects were statistically significant. A repeated-measures ANOVA performed on ethanol intake data showed no significant effects.

3.1.2. CeA infusion of NPY–SAP is associated with a significant reduction of Y1R IR in the CeA

Data representing Y1R IR from mice treated with CeA infusion of NPY–SAP or B-SAP are presented in Table 1. Of the 10 regions that were assessed, the only region that showed a significant reduction of Y1R IR in mice treated with NPY–SAP was the CeA (see Fig. 2 for representative photomicrographs through the CeA). A *t*-test performed in Y1R IR data collected from the CeA was statistically significant [$t(42) = 2.963$, $p = 0.005$] confirming the above conclusion. These observations suggest that the NPY–SAP treat-

Table 1
Y1R IR from mice given neurotoxin injection into the CeA.

Brain region	Y1 immunoreactivity (% area)		
	NPY–SAP treatment	B-SAP treatment	*p* value
Basomedial hypothalamus	0.189 ± 0.026	0.210 ± 0.038	0.648
Paraventricular nucleus of hypothalamus	0.812 ± 0.114	0.919 ± 0.079	0.432
Basolateral amygdala	0.097 ± 0.019	0.164 ± 0.032	0.110
Central nucleus of the amygdala	0.083 ± 0.016	0.262 ± 0.049	0.005*
Medial amygdala	0.176 ± 0.032	0.259 ± 0.052	0.237
Bed nucleus of stria terminalis	0.222 ± 0.050	0.249 ± 0.045	0.691
CA1	0.286 ± 0.028	0.362 ± 0.065	0.304
CA2	0.574 ± 0.033	0.547 ± 0.050	0.667
CA3	0.314 ± 0.029	0.391 ± 0.038	0.202
Dentate gyrus	0.184 ± 0.016	0.243 ± 0.026	0.067

Data are presented as mean ± SEM.
* $p < 0.05$ (two-tailed).

Table 2
Y1R IR from mice given neurotoxin injection into the BMH.

Brain region	Y1 immunoreactivity (% Area)		
	NPY–SAP treatment	B-SAP treatment	*p* value
Basomedial hypothalamus	0.031 ± 0.005	0.076 ± 0.008	0.05*
Paraventricular nucleus of hypothalamus	1.018 ± 0.11	0.919 ± 0.089	0.451
Basolateral amygdala	0.42 ± 0.053	0.287 ± 0.026	0.023*
Central nucleus of amygdala	0.27 ± 0.035	0.236 ± 0.018	0.384
Medial amygdala	0.119 ± 0.007	0.119 ± 0.007	0.940
Bed nucleus of stria terminalis	0.241 ± 0.021	0.247 ± 0.026	0.765
CA1	0.59 ± 0.05	0.647 ± 0.059	0.493
CA2	1.094 ± 0.078	1.026 ± 0.096	0.557
CA3	0.666 ± 0.051	0.535 ± 0.034	0.032*
Dentate gyrus	1.365 ± 0.174	0.967 ± 0.0128	0.051

Data are presented as mean ± SEM.
* $p < 0.05$ (two-tailed).

ment site-specifically killed cells expressing Y1R in the region in which the neurotoxin was injected.

3.2. Experiment 2: BMH infusion of NPY–SAP

3.2.1. BMH infusion of NPY–SAP is associated with decreased anxiety-like behavior

Results from the elevated zero maze and open-field locomotor activity tests in mice treated with BMH infusion of NPY–SAP or B-SAP are presented in Fig. 3. Relative to mice treated with B-SAP ($n = 27$), mice given BMH infusion of NPY–SAP ($n = 21$) showed a significant reduction of anxiety-like behavior, evidenced by significant increases in open area time (Fig. 3A) and number of open area entries (Fig. 3B). *t*-Tests performed on open area time [$t(46) = 3.353$, $p = 0.002$] and open area entries [$t(46) = 2.404$, $p = 0.02$] data both achieved statistical significance. As shown in Fig. 3C, mice treated with BMH infusion of NPY–SAP ($n = 13$) did not show alterations in open-field locomotor activity relative to mice treated with B-SAP ($n = 14$), confirmed by a non-significant *t*-test performed in this dataset [$t(25) = 0.587$, $p = 0.563$]. As above, consummatory measures provide further evidence that NPY–SAP treatment did not impact motor behavior or the health of the mice as there were no significant differences between NPY–SAP ($n = 13$) and B-SAP ($n = 14$) groups in terms of average food

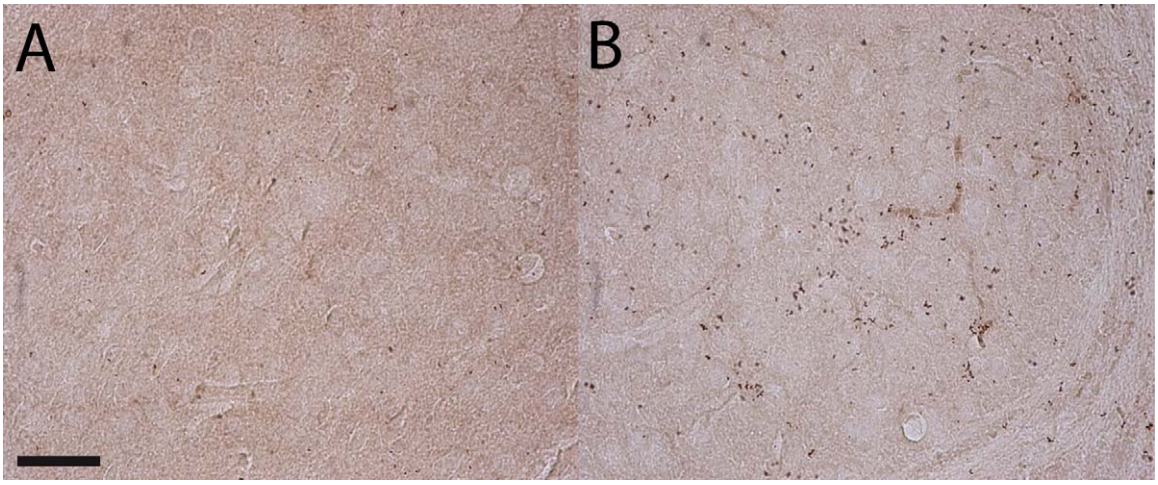

Fig. 2. Representative photomicrographs of 40 μm coronal sections through the CeA showing Y1R IR in mice injected with NPY–SAP (A) or B-SAP (B) into the CeA. Images were photographed and quantified at a magnification of 40×. Scale bar = 50 μm.

intake (328.75 ± 12.24 g/kg/day versus 338.36 ± 11.79 g/kg/day, respectively), water drinking (186.41 ± 9.22 ml/kg/day versus 207 ± 8.88 ml/kg/day, respectively), or ethanol intake (1.14 ± 0.39 g/kg/day versus 1.14 ± 0.38 g/kg/day, respectively). A repeated-measures ANOVA performed on food intake data revealed a significant main effect of week [$F(5, 125) = 14.735$, $p = 0.001$], but no other effects were statistically significant. Repeated-measures ANOVA performed on water and ethanol intake data showed no significant effects.

3.2.2. BMH infusion of NPY–SAP is associated with a significant reduction of Y1R IR in the BMH, but a significant increase in Y1R IR in the BLA and CA3 region in the hippocampus

Data representing Y1R IR from mice treated with BMH infusion of NPY–SAP or B-SAP are presented in Table 2. Of the 10 regions that were assessed, significant alterations of Y1R IR were noted in the BMH, the BLA, and the CA3 region of the hippocampus (see Fig. 4 for representative photomicrographs). A *t*-test performed in Y1R IR data collected from the BMH was statistically significant [$t(51) = 4.553$, $p = 0.001$], reflecting the significant reduction of Y1R IR in NPY–SAP-treated mice. As above, these observations suggest that the NPY–SAP treatment was successful in killing cells expressing the Y1R in the region in which the neurotoxin was injected. Surprisingly, relative to mice treated with B-SAP, mice treated with BMH infusion of NPY–SAP showed a significant compensatory increase in Y1R IR in the BLA [$t(46) = 2.322$, $p = 0.025$] and the CA3 region of the hippocampus [$t(50) = 2.156$, $p = 0.036$].

3.3. Between study comparison of elevated zero maze behavior

We assessed the similarity of anxiety-like behavior in control subjects from the studies involving neurotoxin injection into the CeA versus the BMH. *t*-Tests performed to compare B-SAP groups from each study in terms of open area time [$t(52) = 1.145$, $p = 0.258$] and open area entries [$t(52) = 0.672$, $p = 0.505$] failed to achieve statistical significance, suggesting that the control groups between the studies exhibited similar anxiety-like behavior on the elevated zero maze test. Additionally, when the B-SAP group from the BMH infusion study was compared with the NPY–SAP group from the CeA infusion study, there were significant group differences in terms of open area time [$t(48) = 2.049$, $p = 0.005$] and open area entries [$t(48) = 2.382$, $p = 0.021$], confirming that mice given NPY–SAP injection into the CeA exhibited behavior consistent with increased anxiety. Finally, when the B-SAP group from the CeA infusion study was compared with the NPY–SAP group from the BMH infusion study, there were significant differences between groups in open area time [$t(46) = 2.202$, $p = 0.033$] and open area entries [$t(46) = 3.340$, $p = 0.002$], confirming that mice given NPY–SAP injection into the BMH exhibited behavior consistent with blunted anxiety.

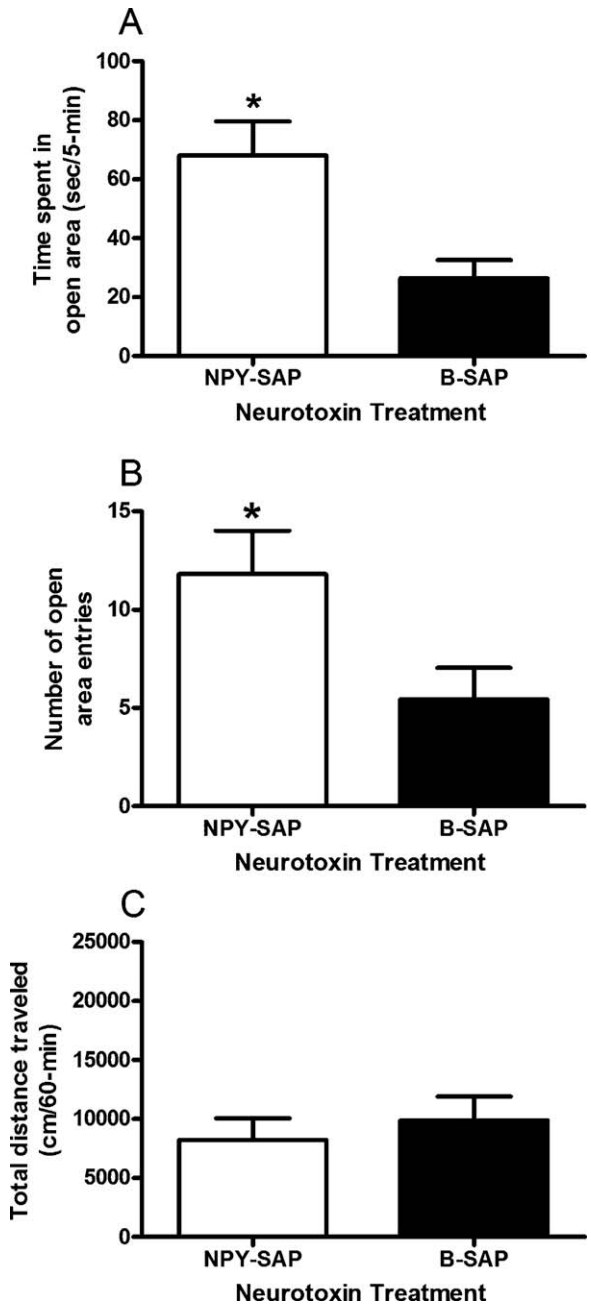

Fig. 3. Results from elevated zero maze and open-field testing in BALB/cJ mice given NPY–SAP or B-SAP injection into the BMH. Relative to B-SAP treated mice, time spent in the open area (A) and number of open area entries (B) were significantly increased in mice treated with NPY–SAP. There were no significant differences between the NPY–SAP and B-SAP groups in open-field locomotor activity (C). $^*p < 0.05$.

4. Discussion

Here we show that there was a significant increase in anxiety-like behavior in BALB/cJ mice injected with the NPY–SAP neurotoxin into the CeA relative to mice treated with the control B-SAP. Thus, CeA NPY–SAP-treated mice spent significantly less time in the open area of the elevated zero maze and made significantly less open arm entries relative to B-SAP-treated mice. Reduced open area activity was not likely related to a general reduction of motor behavior or compromised heath status as there were no significant differences between mice treated with CeA infusion of NPY–SAP or B-SAP in terms of open-field locomotor activity or in measures of consummatory behaviors. Increased anxiety-like behavior was likely the result of blunted NPY signaling in the region of the CeA, as mice treated with NPY–SAP showed a significant reduction of Y1R IR in the CeA relative to B-SAP treated animals. The Y1R IR results reinforce the conclusion that the NPY–SAP toxin successfully lesioned cells in the CeA that express Y1R. CeA-infusion of NPY–SAP site-specifically attenuated Y1R IR in the CeA, and did not significantly alter Y1R IR in nearby regions including the BLA, medial amygdala, and bed nucleus of the stria terminalis. Thus, NPY–SAP appears to be a tool that will allow very precise definition of the NPY neurocircuitry involved in modulating anxiety-like behavior. It is noteworthy that since NPY–SAP binds to all NPY receptors, the NPY–SAP treatment would have also killed Y2- and Y5-expression cells, though we did not quantify these changes in the present report. The observed increase of anxiety-like behavior following CeA injection of NPY–SAP are consistent with results

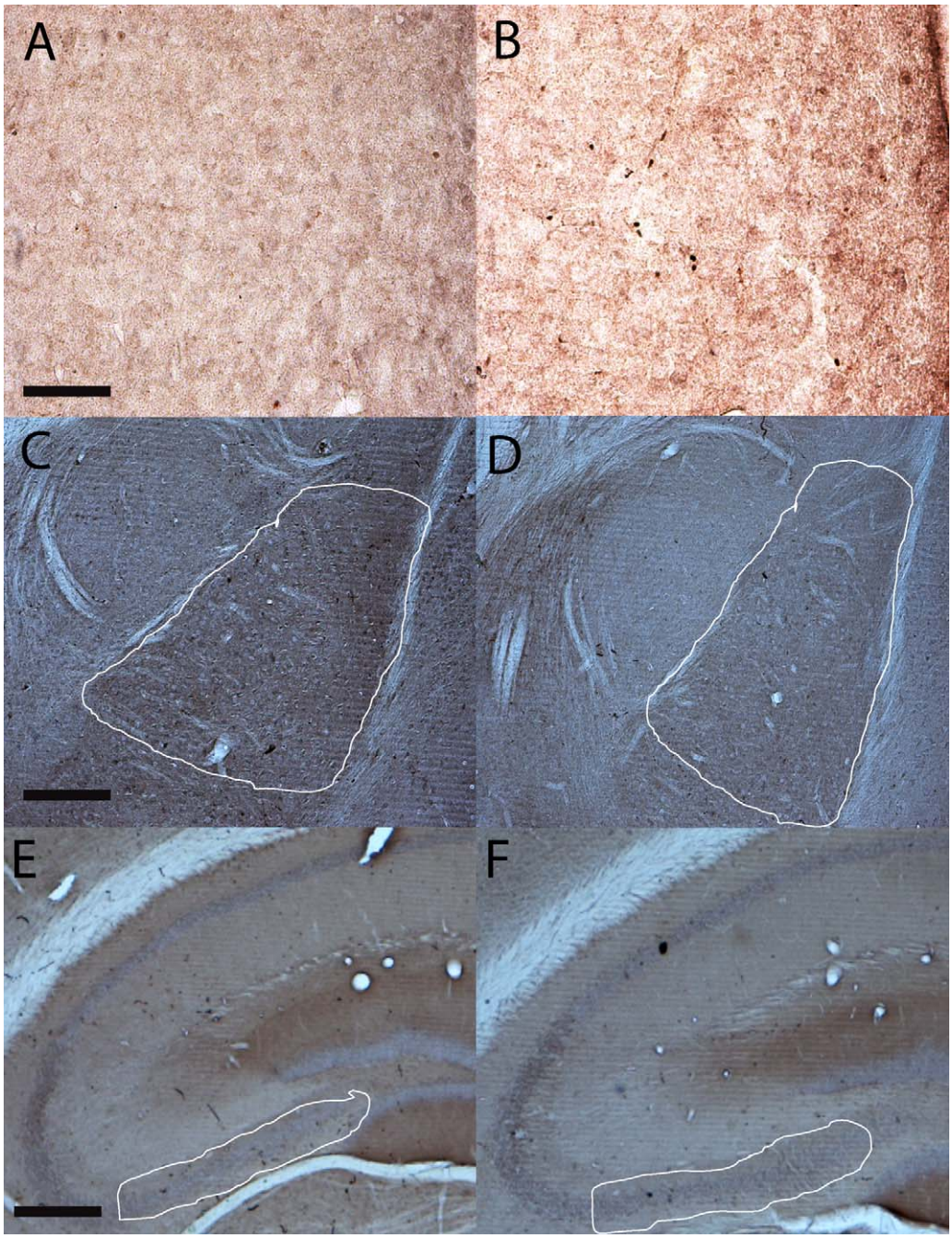

Fig. 4. Representative photomicrographs of 40 μm coronal sections through the BMH (A and B), the BLA (C and D), and CA3 region of the hippocampus (E and F) showing Y1R IR in mice injected with NPY–SAP (A, C, and E) or B-SAP (B, D, and F) into the BMH. Images of the BMH were photographed and quantified at a magnification of 40× (scale bar = 50 μm), while images of the BLA and CA3 were photographed and quantified at a magnification of 10× (scale bar = 200 μm; solid white lines depict the regions that were selected for quantification).

obtained using other tools to blunt NPY signaling such as NPY receptor antagonist and NPY antisense [6,15,20,21]. It should be noted that while NPY signaling in the CeA has been shown to modulate ethanol consumption in rodents [11,20], the lack of an effect of CeA-infused NPY–SAP on ethanol intake here is likely the result of an almost complete avoidance of ethanol in the BALB/cJ mice that were used (only 1.14–1.21 g/kg/day).

As a control, we injected NPY–SAP into the BMH and assessed subsequent anxiety-like behavior. We chose the BMH because, to our knowledge, there is no known link to NPY signaling in this region to the modulation of anxiety-like behavior, and NPY signaling in the hypothalamus appears to be primarily involved in feeding behaviors [3,26,28]. Unexpectedly, mice given BMH injection of NPY–SAP showed reduced anxiety-like behavior relative to B-SAP treated mice, evidenced by increased open area time and open area entries with no associated alterations of open-field locomotor activity or consummatory behaviors. While BMH infusion of NPY–SAP was associated with a significant reduction of Y1R IR in the BMH (confirming the lesioning of Y1R-expressing cells), this treatment also caused a significant increase in Y1R IR in the BLA and the CA3 region of the hippocampus when compared to B-SAP-treated mice. Increased Y1R signaling stemming from an upregulation of Y1R IR in the BLA and/or CA3 region may account for the paradoxical decrease in anxiety-like behavior in mice given

NPY–SAP injection into the BMH. If it is assumed that increased Y1R IR translates into increased NPY signaling in these regions, such increased NPY signaling may have promoted anxiolysis which led to the observed reduction of anxiety-like behavior in the present work. In fact, accumulating evidence indicates that NPY signaling in the BLA and hippocampus protect against anxiety-like behaviors in rodents [17,23,27,29], reinforcing the idea that an upregulation of NPY signaling in these regions accounts for the reduced anxiety-like behavior in mice given BMH infusion of NPY–SAP.

While it is unclear how destruction of Y1R-expressing cells in the BMH confers increases of Y1R IR in other brain regions, it is interesting to note that a previous study that used NPY–SAP to induce lesions of NPY receptor-expression cells in the arcuate nucleus of the hypothalamus observed a significant increase in Y1R IR in other regions including the paraventricular nucleus of the hypothalamus and the perifornical area. Analogous to the present findings, there was a paradoxical increase in feeding behavior in NPY–SAP-treated animals, hypothesized to stem from the compensatory increase in Y1R signaling on other brain regions [5]. Similarly, area postrema lesions were found to be associated with a significant reduction of anxiety-like behavior in rats, an effect that was hypothesized to be related to compensatory increases of NPY mRNA levels in other brain regions that included the amygdala [18]. We suggest that the observed compensatory increase in Y1R IR in the BLA and/or CA3 likely explains the reduced anxiety-like behavior in mice treated with BMH injection of NPY–SAP. Importantly, there are connections between the area of the BMH and the BLA [19] as well as the medial hypothalamus and the hippocampus [1], and these pathways may be involved with the compensatory increases of Y1R IR in the regions outside of the lesion site.

In conclusion, the present set of experiments suggest that the NPY–SAP neurotoxin may be a useful tool for studying the NPY neurocircuitry that modulates anxiety-like behaviors. Consistent with previous work, blunted NPY receptor signaling in the CeA following local injection of NPY–SAP was associated with increased anxiety-like behavior, reinforcing the critical role of NPY signaling in the CeA in the integration of emotional responses. The unexpected reduction of anxiety-like behavior following BMH injection of NPY–SAP may be related to the compensatory increase in Y1R IR in the BLA and/or CA3. This latter observation raises the important point that while NPY–SAP may be a useful tool, caution is necessary when drawing conclusions regarding the role of NPY signaling at the specific lesion site. Characterization of the NPY system in brain regions beyond the site of NPY–SAP injection may be necessary to gain a more accurate picture of the system involved.

Disclosure statement

Both authors disclose that there are no actual or potential financial, personal, or other conflicts of interested related to the work reported in the manuscript.

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The CRF-1 Receptor Antagonist, CP-154,526, Attenuates Stress-Induced Increases in Ethanol Consumption by BALB/cJ Mice

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Background: Corticotropin-releasing factor (CRF) signaling modulates neurobiological responses to stress and ethanol, and may modulate observed increases in ethanol consumption following exposure to stressful events. The current experiment was conducted to further characterize the role of CRF_1 receptor (CRF_1R) signaling in stress-induced increases in ethanol consumption in BALB/cJ and C57BL/6N mice.

Methods: Male BALB/cJ and C57BL/6N mice were given continuous access to 8% (v/v) ethanol and water for the duration of the experiment. When a baseline of ethanol consumption was established, animals were exposed to 5 minutes of forced swim stress on each of 5 consecutive days. Thirty minutes before each forced swim session, animals were given an intraperitoneal injection of a 10 mg/kg dose of CP-154,526, a selective CRF_1R antagonist, or an equal volume of vehicle. The effect of forced swim stress exposure on consumption of a 1% (w/v) sucrose solution was also investigated in an ethanol-naïve group of BALB/cJ mice.

Results: Exposure to forced swim stress significantly increased ethanol consumption by the BALB/cJ, but not of the C57BL/6N, mice. Stress-induced increases in ethanol consumption were delayed and became evident approximately 3 weeks after the first stressor. Additionally, forced swim stress did not cause increases of food or water intake and did not promote delayed increases of sucrose consumption. Importantly, BALB/cJ mice pretreated with the CRF_1R antagonist showed blunted stress-induced increases in ethanol intake, and the CRF_1R antagonist did not influence the ethanol drinking of non-stressed mice.

Conclusions: The present results provide evidence that CRF_1R signaling modulates the delayed increase of ethanol consumption stemming from repeated exposure to a stressful event in BALB/cJ mice.

Key Words: Corticotropin-Releasing Factor, CRF_1 Receptor, Ethanol, Stress, Voluntary Consumption.

STRESS MAY BE a key contributor to the development of ethanol dependence and relapse (Breese et al., 2005; Koob, 2003). Stressful life events, such as those underlying post-traumatic stress disorder, are comorbid with ethanol abuse disorders and human laboratory studies show that stress increases the self-report of craving in abstinent alcoholics (Back et al., 2006; Breslau et al., 2003; Fox et al., 2007). Clinical research implicates stress in the relapse to pathological ethanol use in formerly abstinent alcoholics, perhaps as a means to self-medicate heightened anxiety and negative affect associated with withdrawal and abstinence from alcohol (Brady and Sonne, 1999; Breese et al., 2005; Kushner et al., 1994; Sinha, 2001).

Recent investigations show that stress can also impact ethanol consumption in animal models (Chester et al., 2004; Croft et al., 2005; Le et al., 2000; Little et al., 1999; Liu and Weiss, 2002; Sillaber et al., 2002). Various stress paradigms reliably elicit stress-induced increases in ethanol consumption, especially among low ethanol consuming animals (Chester et al., 2004; Croft et al., 2005; Little et al., 1999). For example, selectively bred ethanol non-preferring NP rats exposed to 10 days of restraint stress showed significant and enduring increases in ethanol consumption beginning approximately 2 weeks following the stress procedure, while ethanol preferring P rats showed only transient stress-induced increases in ethanol drinking immediately after the stress procedure (Chester et al., 2004). Additionally, 3 weeks of stress induced by daily saline injections (Little et al., 1999) or 5 consecutive days of social defeat stress (Croft et al., 2005), significantly increased ethanol consumption approximately 2 weeks after the stress procedure among C57BL/10 mice displaying initially low preference for ethanol. An interesting commonality among many animal studies that assess the effects of stress on ethanol intake is that the effects of stress on ethanol drinking are

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delayed, typically occurring weeks after stress exposure (Chester et al., 2004; Croft et al., 2005; Little et al., 1999).

Both ethanol and stress activate the hypothalamic-pituitary-adrenal (HPA) axis by inducing the release of corticotropin-releasing factor (CRF), adrenocorticotropic hormone (ACTH), and glucocorticoids (Brady and Sonne, 1999). The relationship between ethanol and the HPA-axis appears to be bidirectional, as exogenous administration of CRF, ACTH, and glucocorticoids alter ethanol consumption (Bell et al., 1998; O'Callaghan et al., 2002; Thorsell et al., 2005). Given that neurobiological responses to both stress and ethanol exposure involve HPA-axis signaling, it is possible that the neurochemicals and hormones associated with the HPA-axis modulate stress-induced increases of ethanol consumption. One such candidate is CRF, a 41 amino acid polypeptide that integrates both neuroendocrine and behavioral responses to stress (Smith et al., 1998). CRF-containing neurons are expressed throughout the brain, including in regions implicated in neurobiological responses to ethanol such as the bed nucleus of the stria terminalis, the amygdala, and the lateral hypothalamus (Koob, 2003). Of the two G protein-coupled receptors, the CRF_1 receptor (CRF_1R) appears to be involved with the integrate emotional behavior while the CRF_2 receptor (CRF_2R) may modulate ingestive behaviors (Koob, 2003; Zorrilla and Koob, 2004; Zorrilla et al., 2004).

Corticotropin-releasing factor receptor signaling has been implicated in a variety of neurobiological responses to ethanol. For example, CRF receptor antagonists attenuate the anxiogenic effect of ethanol withdrawal (Breese et al., 2004; Knapp et al., 2004; Overstreet et al., 2004; Rassnick et al., 1993), prevent excessive ethanol self-administration in dependent animals (Funk et al., 2007; Valdez et al., 2002), and block foot shock-induced reinstatement of ethanol-seeking behavior (Liu and Weiss, 2002). The CRF_1R also appears to be involved in stress-induced increases in ethanol consumption. Mutant mice lacking normal production of the CRF_1R displayed significantly greater ethanol consumption beginning approximately 2 weeks after a social defeat stress procedure, an effect that was not evident in normal wild-type mice. Subsequent exposure to forced swim stress further augmented ethanol consumption in CRF_1R knockout mice (Sillaber et al., 2002).

While the Sillaber et al. (2002) study provides genetic evidence suggesting a role for the CRF_1R in modulating stress-induced increases in ethanol consumption, the goal of the present experiment was to use a pharmacological approach to determine if pretreatment with the selective CRF_1R antagonist, CP-154,526, would buffer the effects of stress and thus attenuate the development of stress-induced increases in ethanol intake in BALB/cJ mice. Therefore, we predicted that (1) ethanol consumption would increase among animals with a history of stress exposure and (2) pretreatment with CP-154,526 would attenuate stress-induced increases in ethanol consumption among animals with a history of stress. BALB/cJ mice were chosen because this strain has been shown to have high sensitivity to the effects of stress on both behavioral and neurobiological measures (Crawley et al., 1997) and drinks low levels of ethanol (Belknap et al., 1993). We also assessed the effects of stress exposure on ethanol consumption by C57BL/6N mice, a strain that voluntarily consumes high amounts of ethanol (Belknap et al., 1993). Here we show that 5 consecutive days of exposure to a 5-minute forced swim stress procedure caused significant and delayed increases in voluntary ethanol consumption in BALB/cJ mice, an effect which was attenuated by pretreatments with the CRF_1R antagonist before each stress session. On the other hand, stress exposure did not alter ethanol intake by C57BL/6N mice.

MATERIALS AND METHODS

Animals

Forty-seven male BALB/cJ (Jackson Laboratories, Bar Harbor, ME) and 36 male C57BL/6N (Charles River Labs, Wilmington, MA) mice approximately 8-week old and weighing 19 to 26 g were housed individually in polypropylene cages with corncob bedding upon arrival. Animals had ad libitum access to tap water and standard rodent chow throughout the experiment. All fluid was presented in 2 bottles, inserted through holes at the top of the cage. Bottle weights were recorded every 2 days, and body weights and food measurements were taken every 4 days at approximately 10:00 AM. Food intake was measured by subtracting the weight of rodent chow (grams) still present in the cage on measurement day from the initial weight when food was placed in the cage. Great care was taken to collect the remaining food in the cage on measurement day to assure accurate readings. The colony room was maintained at approximately 21° C with a 12-h/12-h light/dark cycle with lights off at 10:30 AM. All procedures in the experiments below were approved by the University of North Carolina's Institutional Animal Care and Use Committee and follow the National Institute of Health's guidelines.

Drug Treatment

CP-154,526 (butyl-[2,5-dimethyl-7-(2,4,6-trimethylphenyl)-7H-pyrrolo[2,3-d]pyrimidin-4-yl]-ethylamine) donated by Pfizer (Groton, CT) was suspended in a vehicle of 0.5% carboxymethylcellulose (CMC). CP-154,526 displays high affinity for the CRF_1R (K_i < 10 nM) and blocks CRF-stimulated adenylate cyclase activity in rodent pituitary and cortical membranes (Lundkvist et al., 1996; Schulz et al., 1996). Peripheral administration of CP-154,526 crosses the blood–brain barrier and reaches peak brain concentrations 20 minutes after administration with significant levels of the drug observed in the cortex, striatum, cerebellum, and hippocampus (Keller et al., 2002). Importantly, previous research found that systemic administration of a 10 mg/kg dose of CP-154,526 effectively reduced anxiety-like behavior in mice (Griebel et al., 1998). Therefore, a 10 mg/kg dose of CP-154,526, or equal volume of CMC (5 ml/kg), was administered via intraperitoneal (i.p.) injection approximately 30 minutes prior to each stress or handling procedure (see below).

Forced Swim Stress

Forced swim procedures were used to induce stress in mice. Briefly, the mice were removed from their homecages and placed individually in buckets containing 4,000 ml of water maintained at approximately

room temperature (21°C) for 5 minutes on each of 5 consecutive days. Mice were carefully monitored and a criteria was established that any mouse that could not keep its head above the water was removed from the procedure (however, all animals were able to swim for the entire session in each experiment). After the 5-minute session, mice were removed from the buckets and dried with a cloth towel. This forced swim stress procedure has been shown to significantly increase ethanol drinking by mice (Sillaber et al., 2002). Mice in the non-stress conditions were briefly removed and then returned to their cages.

Habituation to Environment and Voluntary Ethanol Consumption

Upon arrival, animals were allowed to habituate to their surroundings for 8 days. On day 9, 1 water bottle on each cage was replaced with an identical bottle containing a 2% (v/v) ethanol solution diluted in tap water. Every 4 days, the concentration of ethanol was increased in the following increments: 4, 6, and 8%. From this point on, animals had continuous free access to 8% ethanol and water for the duration of the experiment. The position of bottles containing ethanol were changed every 2 days to prevent the development of side preferences. Fluid loss was controlled by using dummy bottles of water and ethanol placed on an animal-free cage which was located on the same rack as cages containing mice. Daily ethanol consumption was calculated in grams of ethanol consumed/kg of body weight (g/kg).

Consumption of the 8% ethanol solution stabilized by day 13, and animals were divided into 4 groups based on ethanol consumption during the final 3 days of baseline (days 16 to 18). Mice were either pretreated with CP-154,526 (CP) or vehicle (Veh) 30 minutes before being exposed to a 5-minute forced swim stress session (Stress) or handling (No Stress). The groups were as follows: BALB/cJ Stress-CP ($n = 8$), BALB/cJ Stress-Veh ($n = 8$), BALB/cJ No Stress-CP ($n = 9$), BALB/cJ No Stress-Veh ($n = 9$), C57BL/6N Stress-CP ($n = 10$), C57BL/6N Stress-Veh ($n = 7$), C57BL/6N No Stress-CP ($n = 9$), and C57BL/6N No Stress-Veh ($n = 10$). Following the 5-forced swim days, ethanol, water, and food intake as well as body weight measures were collected over a 4-week period. The BALB/cJ mice were exposed to an additional 5 days of forced swim stress on days 56 to 60, as described above, but did not receive drug treatment prior to stress exposure.

Voluntary Sucrose Consumption and Forced Swim Stress

As a consummatory control, 20 ethanol-naïve BALB/cJ mice were given continuous access to a 1% (w/v) sucrose solution and tap water and exposed to forced swim stress or handling, as described above. Sucrose was diluted in tap water. We chose 1% sucrose because we found that this concentration produced a similar volume of consumption by the BALB/cJ mice as the 8% ethanol solution. Additionally, 1% sucrose solution has been used previously as a control for stress-induced consumption of an 8% ethanol solution (Croft et al., 2005). The position of bottles containing sucrose was changed every 2 days to prevent the development of side preferences. Fluid loss was controlled by using dummy bottles of water and sucrose placed on an animal-free cage which was located on the same rack as cages containing mice. Daily sucrose consumption was calculated in milliliters of sucrose solution consumed/kg of body weight (ml/kg). Access to food, water, and sucrose was continuously available for the duration of the experiment.

Following 7 days of access to the 1% sucrose solution, animals were divided into Stress and No Stress groups based on their sucrose consumption during the final 3 days of baseline (days 5 to 7). On days 8 through 12, animals in the Stress group ($n = 10$) were exposed to daily 5-minute forced swim procedures over 5 days, while animals in the No Stress group ($n = 10$) were handled as described above. Sucrose and water consumption were monitored every 2 days throughout the stress period, and for an additional 4 weeks thereafter.

Data Analyses

All data shown are presented as means ± SEM and were analyzed using repeated measures analyses of variance (ANOVAs). Planned comparisons were analyzed using *t*-tests (Winer, 1991). In accordance with a priori hypotheses, the following tests were conducted: (1) comparisons were made of the Stress-Veh and No Stress-Veh groups to determine if stress exposure significantly increased ethanol consumption, (2) comparisons were made of the Stress-CP group with No Stress-CP and No Stress-Veh groups to determine if CP-154,526 pretreatment significantly attenuated stress-induced ethanol drinking to the level of non-stressed animals, and (3) comparisons were made of the Stress-Veh and Stress-CP groups to determine if CP-154,526 pretreatment significantly blocked stress-induced increases of ethanol drinking relative to stressed animals not pretreated with the CRF_1R antagonist. All reports of significance were accepted at the $p < 0.05$ level.

RESULTS

Figure 1 displays the effect of forced swim stress on the ethanol, water, and food consumption of BALB/cJ animals for the duration of the experiment. Because BALB/cJ mice were treated with the CRF_1R antagonist during the first, but not second, 5 day stress procedure, data were collapsed across the CRF_1R antagonist factor for the present analyses. As shown in Fig. 1*A*, forced swim stress significantly increased ethanol consumption among BALB/cJ animals in the Stress group, while handling did not alter ethanol consumption among BALB/cJ animals in the No Stress group. The results of a 2×11 repeated measures ANOVA revealed a significant main effect of week [$F(10,340) = 4.859$], a significant stress × week interaction [$F(10,340) = 2.634$], as well as a significant main effect of stress [$F(1,34) = 8.315$]. Planned comparisons revealed that stressed animals consumed significantly more ethanol than non-stressed animals at post-stress week 3 [$t(34) = 2.503$] and post-stress week 4 [$t(34) = 2.697$] following the first stressor. Additionally, stressed animals consumed significantly more ethanol during the second baseline period [$t(34) = 2.271$], during the second stress period [$t(34) = 1.971$], and at post-stress week 1 [$t(34) = 2.001$], post-stress week 2 [$t(34) = 2.378$], and post-stress week 3 [$t(34) = 2.845$] following the second stressor. Animals of the Stress group consumed significantly less water when compared with animals of the No Stress group for much of the experiment (see Fig. 1*B*). The results of a 2×11 repeated measures ANOVA revealed a significant main effect of week [$F(10,340) = 5.750$] and a significant stress × week interaction [$F(10,340) = 3.342$]. Planned comparisons revealed that animals of the Stress group consumed significantly less water than animals of the No Stress group at post-stress week 4 following the first stressor [$t(34) = 2.423$] and following the second stressor at post-stress week 1 [$t(34) = 1.733$], post-stress week 2 [$t(34) = 2.234$], and post-stress week 3 [$t(34) = 1.727$]. The decrease in water consumption among

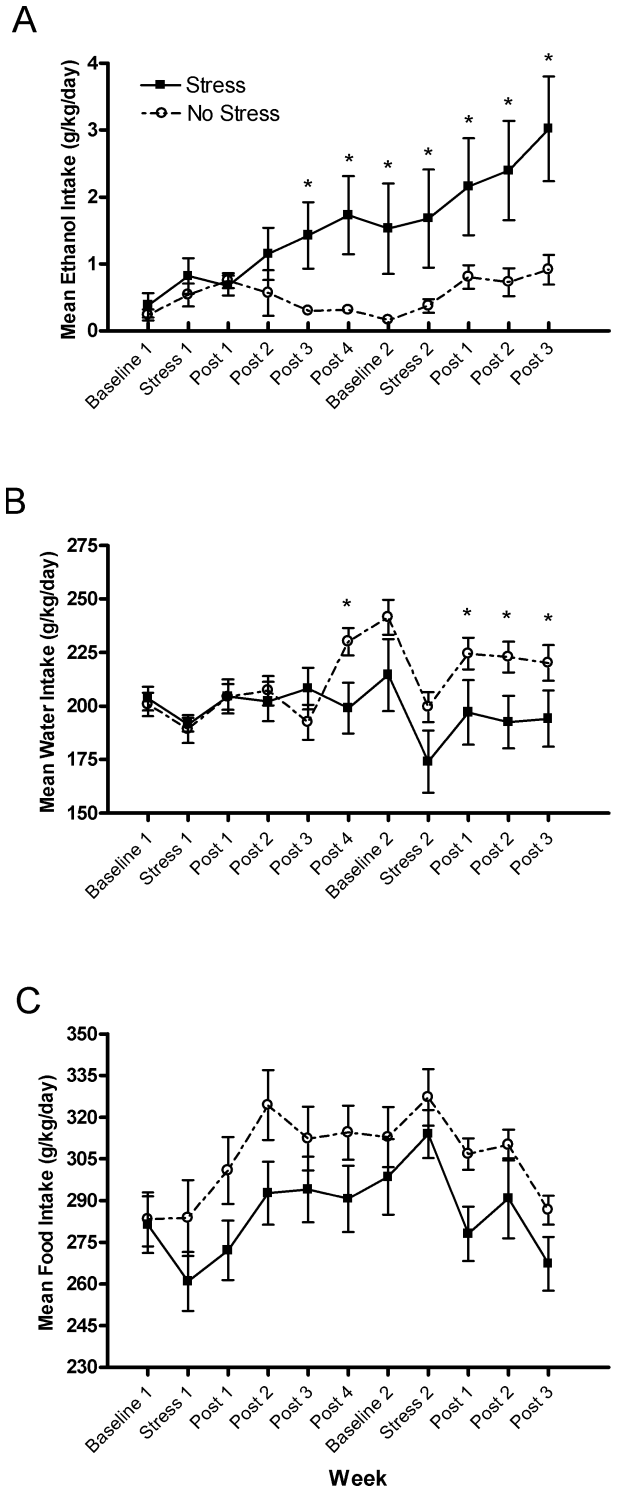

Fig. 1. Mean consumption (g/kg/d) of (**A**) ethanol, (**B**) water, and (**C**) food during baselines, the first and second stressors, and post-stress periods for BALB/cJ Stress and No Stress groups. All values are means ± SEM and *denotes significant between-group differences at the $p < 0.05$ level.

stressed animals is likely related to increased ethanol consumption following stress exposure. Finally, forced swim stress did not alter food consumption when compared with the handled group (see Fig. 1*C*), although a 2×11 repeated measures ANOVA revealed a significant main effect of week [$F(10,320) = 7.162$].

Figure 2 shows the effect of CRF_1R antagonism on ethanol, water, and food consumption of BALB/cJ animals during the first stress period. As shown in Fig. 2*A*, forced swim stress significantly increased ethanol consumption, an effect which was attenuated by administration of CP-154,526. The results of a $2 \times 2 \times 6$ repeated measures ANOVA indicated a significant stress × week interaction [$F(5,160) = 2.979$] as well as a significant main effect of stress [$F(1,32) = 17.986$]. Planned comparisons revealed that animals of the Stress-Veh group consumed significantly more ethanol than animals of the No Stress-Veh groups at post-stress week 3 [$t(16) = 2.046$] and post-stress week 4 [$t(16) = 1.963$], indicating stress-induced increases of ethanol consumption. Importantly, at no time point did group Stress-CP differ significantly from the non-stressed groups.

As stress-induced increases in ethanol consumption emerged several weeks following the stress procedure, the effects of CRF_1R antagonism on the development of stress-induced increases in ethanol consumption were analyzed by examining ethanol consumption at post-stress weeks 2 to 4 relative to the first week following the stress procedure (Δ post 1; see Fig. 2*B*). The results of a $2 \times 2 \times 3$ repeated measures ANOVA revealed a significant main effect of stress [$F(1,32) = 12.232$]. Planned comparisons revealed that animals of the Stress-Veh group showed significantly greater increases of ethanol consumption compared with the No Stress-Veh group at post-stress week 3 [$t(16) = 2.293$] and post-stress week 4 [$t(16) = 2.249$], again reflecting a delayed stress-induced increase in ethanol consumption. A planned comparison revealed significant differences between the Stress-Veh and Stress-CP groups at post-stress week 2 [$t(14) = 1.782$], suggesting that CP-154,526 blocked stress-induced increases in ethanol consumption during this week. As above, at no time point did the Stress-CP group differ significantly from the non-stressed groups.

Exposure to forced swim stress significantly altered water consumption, as displayed in Fig. 2*C*. The results of a $2 \times 2 \times 6$ repeated measures ANOVA revealed a significant main effect of week [$F(5,160) = 5.514$] as well as a significant stress × week interaction [$F(5,160) = 2.853$]. Planned comparisons revealed that the Stress-Veh group consumed significantly less water than the No Stress-Veh group at post-stress week 4 [$t(16) = 2.026$]. Finally, neither forced swim stress nor antagonism of the CRF_1R altered food consumption (see Fig. 2*D*). However, a significant main effect of week was observed [$F(5,160) = 7.486$].

Figure 3 shows the effects of forced swim stress on consumption of the 1% sucrose solution and water by ethanol-naive BALB/cJ mice. Repeated measures ANOVA did not reveal significant effects of stress on sucrose consumption when expressed as ml/kg/d or change in consumption relative to post-stress week 1. However, planned comparisons revealed significant differences in sucrose consumption

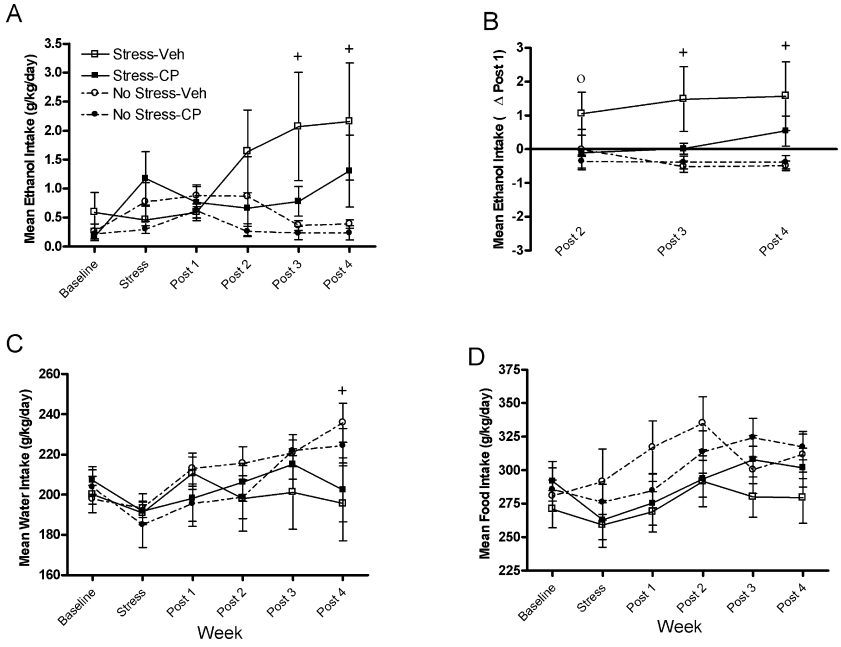

Fig. 2. (**A**) Mean ethanol consumption (g/kg/d) during the first baseline, stressor, and post-stress periods for BALB/cJ mice. (**B**) Mean changes in ethanol consumption (g/kg/d) during post-stress weeks 2 to 4 relative to post-stress week 1 during the first post-stress period for BALB/cJ mice. (**C**) Mean water consumption (g/kg/d) during the first baseline, stressor, and post-stress periods for BALB/cJ mice. (**D**) Mean food consumption (g/kg/d) during the first baseline, stressor, and post-stress periods for BALB/cJ mice. Groups are as follows: Stress-Veh = mice pretreated with vehicle prior to forced swim exposure; Stress-CP = mice were pretreated with CP-154,526 prior to forced swim exposure; No Stress-Veh = mice were treated with vehicle and handled; No Stress-CP = mice were treated with CP-154,526 and handled. All values are means ± SEM. The high degree of variance noted in group Stress-Veh reflects an increase of random variation. Significant between group differences are as follows: ○denotes significant differences between the Stress-Veh and Stress-CP groups and +denotes significant differences between the Stress-Veh and No Stress-Veh groups, at the $p < 0.05$ level.

between groups. Specifically, as shown in Fig. 3*A*, significant differences in sucrose consumption were observed in stressed animals when compared with non-stressed animals at post-stress week 3 [$t(17) = 1.884$], and at post-stress week 4 [$t(17) = 2.139$], which appears to reflect a reduction of sucrose consumption by non-stressed mice at post-stress weeks 3 and 4 relative to prior weeks. Importantly, forced swim stress did not cause a delayed increase in sucrose consumption at post-stress weeks 2 to 4 relative to post-stress week 1 (Δ post 1). The effects of forced swim stress exposure on water consumption are shown in Fig. 3*C*. A 2×6 repeated measures ANOVA revealed a significant main effect of week [$F(5,85) = 6.237$], and planned comparisons revealed that the stressed animals consumed significantly less water than non-stressed animals at post-stress week 3, [$t(17) = 1.829$].

Figure 4 displays the effects of forced swim stress and CRF_1R antagonism on the ethanol and water consumption of C57BL/6N animals. As shown in Fig. 4*A*, neither forced swim stress nor CRF_1R antagonism significantly altered ethanol consumption by C57BL/6N animals. A $2 \times 2 \times 6$ repeated measures ANOVA revealed a significant main effect of week [$F(5,160) = 20.425$]. Planned comparisons revealed no group differences. Figure 4*B* shows water consumption by C57BL/6N mice. The results of a $2 \times 2 \times 6$ repeated measures ANOVA revealed a significant main effect of week [$F(5,160) = 7.087$], as well as a significant week × stress × drug interaction [$F(5,160) = 2.561$]. Planned comparisons revealed that animals of the Stress-Veh group consumed significantly more water than animals of the No Stress-Veh group at post-stress week 1 [$t(17) = 1.789$].

DISCUSSION

The results of the current experiment show that forced swim stress induced a delayed increase in ethanol consumption by initially low ethanol consuming BALB/cJ mice, but did not affect ethanol consumption in the initially high ethanol consuming C57BL/6N mice. The lack of effect of stress exposure on ethanol consumption by the C57BL/6N mice is unlikely due to the high baseline ethanol consumption observed in these animals (e.g., a ceiling effect) as experimental manipulations, such as procedures that promote the alcohol deprivation effect, have been shown to reliably increase ethanol consumption significantly above baseline levels which are similar to consumption levels observed in the present experiment (Melendez et al., 2006). These results are consistent with the literature suggesting that a variety of stressors can have delayed effects on ethanol consumption in rodents (Chester et al., 2004; Croft et al., 2005; Little et al., 1999; Sillaber et al., 2002), and that the effects of stress on ethanol consumption may depend on initial preference for ethanol (Chester et al., 2004; Little et al., 1999; Rockman et al., 1987). The results of the current experiment also provide additional support for research suggesting that CRF_1R signaling is

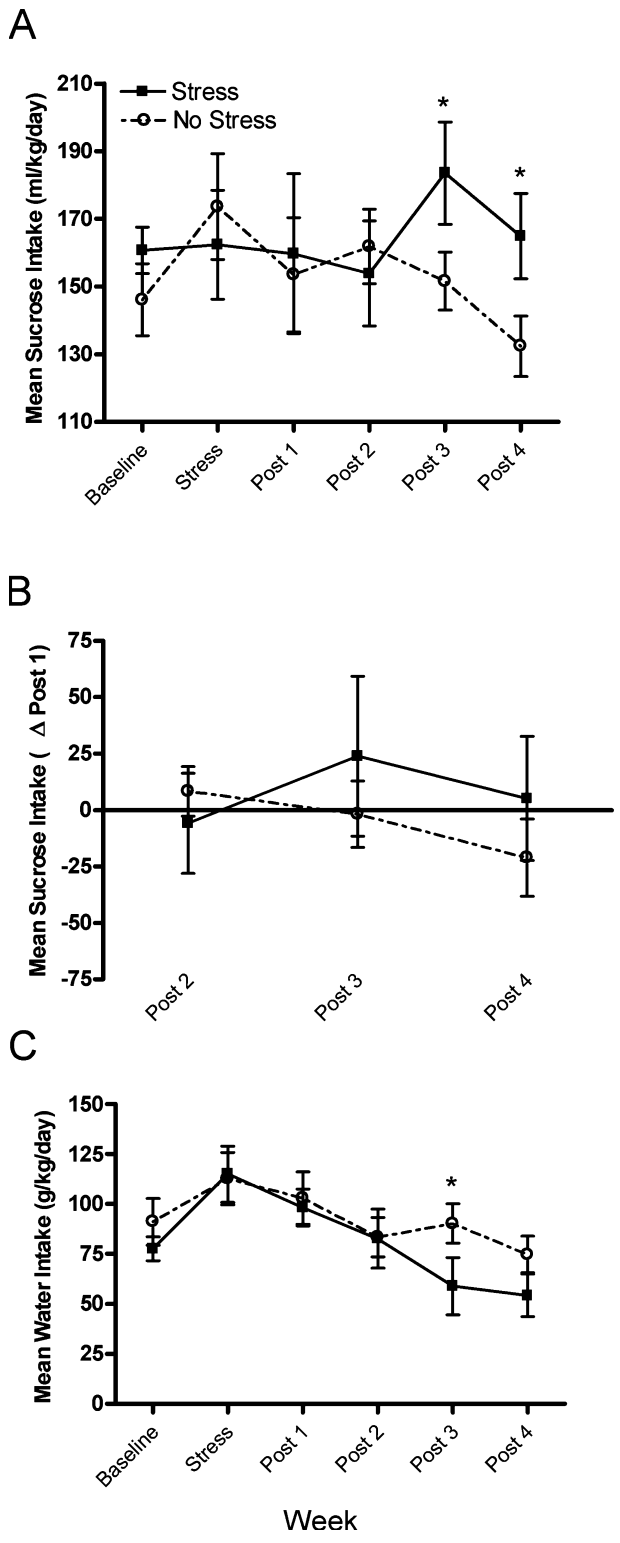

Fig. 3. (**A**) Mean consumption (ml/kg/d) of a 1% (w/v) sucrose solution during the baseline, stress, and post-stress periods for BALB/cJ Stress and No Stress groups. (**B**) Mean change in sucrose consumption (ml/kg/d) during post-stress weeks 2 to 4 relative to post-stress week 1 for BALB/cJ Stress and No Stress groups. (**C**) Mean water consumption (g/kg/d) during the baseline, stress, and post-stress period for BALB/cJ Stress and No Stress groups. All values are means ± SEM, and *denotes significant differences between the Stress and No Stress groups, at the $p < 0.05$ level.

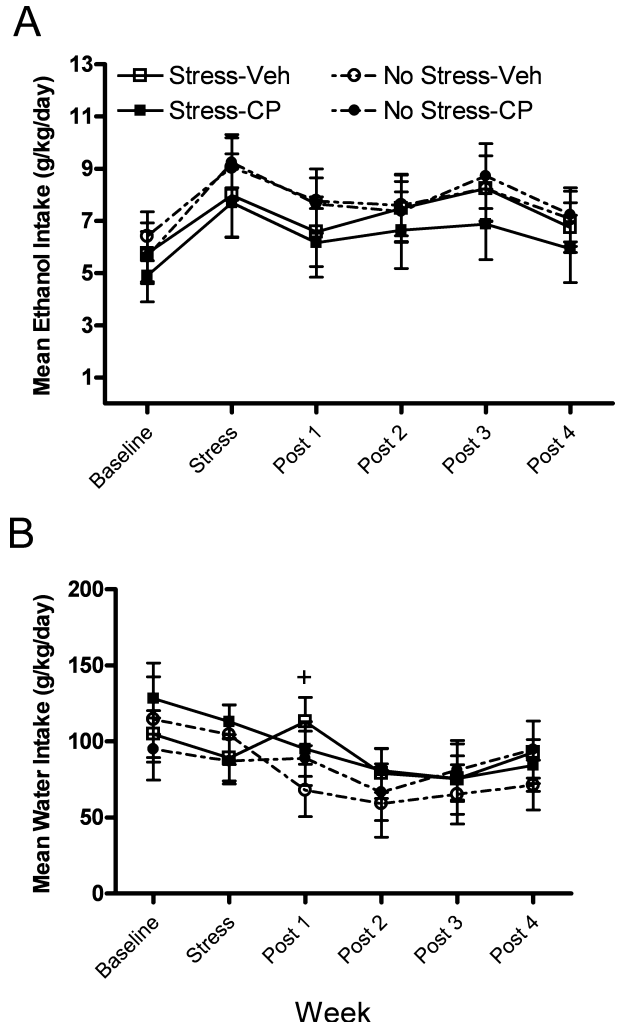

Fig. 4. (**A**) Mean consumption (g/kg/d) of ethanol during the baseline, stress, and post-stress periods for C57BL/6N mice. (**B**) Mean water consumption (g/kg/d) during the baseline, stress, and post-stress periods for C57BL/6N mice. Groups are as follows: Stress-Veh = mice pretreated with vehicle prior to forced swim exposure; Stress-CP = mice were pretreated with CP-154,526 prior to forced swim exposure; No Stress-Veh = mice were treated with vehicle and handled; No Stress-CP = mice were treated with CP-154,526 and handled. All values are means ± SEM, and $^{+}$denotes significant differences between the Stress-Veh and No Stress-Veh groups at the $p < 0.05$ level.

involved in stress-related ethanol consumption as pretreatment before each stress episode with CP-154,526, a CRF_1R antagonist, attenuated the observed stress-induced increases in ethanol consumption among BALB/cJ mice. This conclusion is supported by the observation that stress-treated BALB/cJ mice that were pretreated with CP-154,526 never differed significantly in ethanol consumption from non-stressed groups, while stress-treated mice pretreated with the vehicle showed significantly higher levels of ethanol consumption than the non-stressed groups at multiple time points.

Although there were group differences in sucrose consumption, such differences appear to be related, in part, to a reduction of sucrose intake by non-stressed mice at post-stress weeks 3 and 4 relative to prior weeks. Furthermore, there

were no group differences in sucrose consumption at post-stress weeks 2 through 4 relative to post-stress week 1, indicating that stress did not promote a delayed increase of sucrose consumption, a delayed effect of stress that was noted when mice drank ethanol. This observation, and the fact that stress did not significantly alter food intake, suggests that the delayed effect of stress to increase consumption over weeks is specific to ethanol. The observed decrease in water consumption among animals exposed to stress is likely related to the observed increase in ethanol solution intake among these animals, as a portion of the animal's water intake was obtained from the ethanol solution.

Although the literature on stress and ethanol consumption has been mixed, recent reports indicate that the effects of stress on ethanol consumption may differ depending on the length of time that has elapsed since termination of the stressor. For example, some studies investigating the immediate effects of stress on ethanol consumption suggest that ethanol consumption is transiently reduced (van Erp and Miczek, 2001), and some studies investigating the long-term effects of stress on ethanol consumption reveal delayed increases in ethanol consumption (Chester et al., 2004; Croft et al., 2005; Sillaber et al., 2002), though other studies have failed to find a stress effect on ethanol consumption at any experimental time point (Bowers et al., 1997; Boyce-Rustay et al., 2007). Indeed, direct comparison of the results of these studies is difficult due to use of a wide variety of stressors and rodent strains, as well as varying experimental time points and ethanol access periods. Nonetheless, our work and the work of others indicate that stress can increase ethanol consumption by rodents under certain conditions.

The results of the current experiment coincide with an increasing number of reports suggesting that the pattern of ethanol consumption following stress may be dependent on predisposed ethanol preference (Chester et al., 2004; Little et al., 1999; Rockman et al., 1987), as increases in ethanol consumption were observed in initially low ethanol consuming BALB/cJ mice approximately 3 weeks after exposure to forced swim stress, but not in initially high ethanol consuming C57BL/6N mice. Prior research suggests that animals genetically predisposed, or phenotypically selected, for high ethanol consumption, such as the C57BL/6 strain of mice, reduce ethanol consumption during stress exposure and gradually return to baseline levels of consumption after termination of the stressor (Chester et al., 2004; Rockman et al., 1987). For example, ethanol preferring P rats displayed significantly reduced ethanol consumption during the first 5 days of exposure to 10 days of unpredictable restraint stress, an increase in ethanol consumption during the 5 days immediately following the termination of the restraint stress, and a subsequent return to baseline levels of ethanol consumption (Chester et al., 2004). Similarly, Wistar rats screened for high ethanol preference and exposed to unpredictable restraint stress at cold temperatures significantly reduced their ethanol consumption during the first 12 days of an 18-day stress period, after which consumption returned to baseline levels (Rockman et al., 1987).

Conversely, a variety of observations reveal that animals showing initial low ethanol preference, such as the BALB/c strain of mice, continue consuming baseline levels of ethanol during, and immediately following stress exposure, but increase levels of ethanol consumption approximately 2 to 3 weeks following termination of the stressor (Chester et al., 2004; Croft et al., 2005; Rockman et al., 1987). Consistently, ethanol non-preferring NP rats exposed to 10 days of unpredictable restraint stress maintained baseline levels of ethanol consumption throughout the stress period and immediately thereafter, and significantly increased ethanol consumption approximately 2 weeks following stress exposure (Chester et al., 2004). Wistar rats screened for low ethanol preference and exposed to 18 days of unpredictable restraint stress at cold temperatures displayed gradual increases in ethanol consumption beginning in the final 12 days of the stress period and continuing several weeks after the stress exposure (Rockman et al., 1987). Similar delayed increases in ethanol consumption have been observed in C57BL/10 mice screened for low ethanol preference and exposed to social defeat stress (Croft et al., 2005), and stress caused by repeated saline injections (Little et al., 1999; O'Callaghan et al., 2002). Thus, an emerging literature provides converging evidence that a variety of stressors induce delayed increases in ethanol consumption in initially low ethanol consuming animals. While the present observations provide additional evidence that stress-induced increases in ethanol drinking are evident in low (BALB/cJ), but not high (C57BL/6N), ethanol preferring strains, an alternative explanation for the present data is that the BALB/cJ mice were more stress-responsive than the C57BL/6N mice. Indeed, a well-established literature suggests that the BALB/c strain of mice display higher levels of anxiety and are more stress-responsive on certain behavioral measures than the C57BL/6 strain of mice (Anisman et al., 2007; Carola et al., 2002; Crawley et al., 1997; Depino and Gross, 2007; Ducottet and Belzung, 2004; Griebel et al., 2000). As such, it may be stress sensitivity, rather than initial ethanol preference, that predicts the effects of stress on subsequent ethanol intake.

The HPA-axis has been implicated in neurobiological responses to stress and ethanol consumption, and the involvement of neurochemicals and hormones associated with the HPA-axis in stress-induced ethanol consumption has been demonstrated. For example, Sprague–Dawley rats with intact HPA-axis function displayed increases in ethanol consumption following 11 days of unpredictable exposure to either isolation or immobilization stress, while the post-stress ethanol consumption of hypophysectomized rats did not change (Nash and Maickel, 1988). Pharmacological manipulations also provide evidence for a role of HPA-axis signaling. ACTH administered via unpredictable, i.v. injections for 11 days in intact rats produced increases in ethanol consumption similar to those observed following stress exposure (Nash and Maickel, 1988). Mice screened for low ethanol preference and given

3 weeks of daily i.p. injections of the corticosterone synthesis inhibitor metyrapone did not display stress-induced increases in ethanol preference caused by repeated i.p. injection, while mice injected with vehicle over 3 weeks did display increases in ethanol preference (O'Callaghan et al., 2002). The Type II glucocorticoid receptor appears to modulate the effects of corticosterone on stress-induced increases in ethanol consumption as mice screened for low ethanol preference and given daily i.p. injections of the glucocorticoid Type II receptor antagonist RU38486 did not display stress-induced increases in ethanol preference, an effect observed in mice with low ethanol preference and given daily i.p. injections of vehicle (O'Callaghan et al., 2002).

The results of the current experiment, as well as those of Sillaber et al. (2002), indicate that CRF signaling, via the CRF_1R, is another HPA-axis-associated neurochemical that modulates stress-induced ethanol consumption. In the current experiment, the role of the CRF_1R was investigated pharmacologically through the administration of the CRF_1R antagonist CP-154,526 prior to each exposure to forced swim stress. While only 1 dose of the CRF_1R antagonist was used in the present study, this 10 mg/kg dose of CP-154,526 has been previously shown to reduce anxiety-like behavior in BALB/cJ mice (Griebel et al., 1998). Importantly our results indicate that pharmacological antagonism of the CRF_1R with a 10 mg/kg dose of CP-154,526 attenuates the delayed stress-induced increases in ethanol consumption observed in vehicle and stress treated animals. On the other hand, Sillaber et al. (2002) found that disruption of CRF_1R signaling by genetic mutation augmented the delayed stress-induced increases of ethanol consumption relative to wild-type mice. While the factors that contribute to the inconsistencies between pharmacological and genetic manipulation of CRF_1R signaling are not completely clear, Sillaber et al. (2002) suggest that the observed increases in ethanol consumption among CRF_1R knockout mice following stress exposure may result from developmental compensation associated with mutation of the CRF_1R gene. It should be noted that although the results of the current experiment suggest that the CRF_1R modulates stress-related ethanol consumption, it remains unclear if CRF_1R signaling within the HPA-axis and/or within extrahypothalamic brain regions are involved. In fact, a recent report found that pretreatment with the CRF_1R antagonist antalarmin attenuated yohimbine-induced increases in ethanol self-administration in rats without altering yohimbine-induced increases of corticosterone levels, suggesting that extrahypothalamic CRF_1R signaling was involved (Marinelli et al., 2007).

In summary, the current experiment indicates that exposure to stress is associated with delayed increases in ethanol consumption among initially low consuming BALB/cJ mice, but not initially high consuming C57BL/6N mice. Importantly, stress did not alter the consumption of food or cause delayed increases of sucrose intake in BALB/cJ mice. Pretreatment before each stress episode with the CRF_1R antagonist CP-154,526 attenuated the delayed increases in ethanol consumption observed in stressed BALB/cJ mice, but did not alter the consumption of ethanol by non-stressed mice. Current research indicates that CRF signaling, via the CRF_1R, is intricately involved in the development of ethanol dependence and relapse to ethanol seeking during abstinence (Heilig and Koob, 2007), perhaps due to the role CRF plays in mediating increased anxiety during withdrawal from ethanol (Breese et al., 2004). The current experiment supports the hypothesis that CRF, and more specifically the CRF_1R, is also involved in delayed and long-lasting stress-induced increases in ethanol drinking. Thus targets aimed at the CRF_1R may be useful compounds for treating and/or preventing the lasting effects of stress exposure to induce excessive and uncontrolled ethanol consumption in the human population. Finally, future research will extend the current findings by investigating the role of CRF_1R signaling in targeted brain areas, as well as the role of CRF in stress-induced ethanol drinking by ethanol dependent animals.

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THE NPY Y2 ANTAGONIST BIIE0246 BLOCKS THE EXPRESSION OF AN ALCOHOL DEPRIVATION EFFECT IN ALCOHOL PREFERRING (P) RATS

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The neuropeptide Y Y2 receptor is located both pre- and post-synaptically and functions in an autoreceptor loop mechanism. Previous research has demonstrated attenuation of ethanol consumption in non-selected rats following intracerebroventricular (ICV) administration of the Y2 antagonist BIIE0246. The purpose of the present investigation was to determine the effects of BIIE0246 on ethanol intake in selectively bred P rats undergoing an alcohol deprivation effect (ADE), the temporary increase in ethanol intake observed following ethanol reinstatement after a period of imposed abstinence. Female P rats had continuous 24-hr access to 10% v/v ethanol and water. The ADE group (n=28) had 6 weeks of ethanol drinking followed by a 14 or 16 day period of ethanol deprivation during which rats were implanted with cannulae into the lateral ventricles. Half of the rats received ICV infusions of BIIE0246 (0.5 nmol) while the remaining rats received infusions of the drug vehicle immediately before reinstatement of ethanol availability. A second, continuous access group (n=27) was treated in a parallel manner except that their ethanol access was uninterrupted. Ethanol, water, and food intake was measured at 2 and 24 hr following infusions. Vehicle infused rats in the ADE group showed increased ethanol intake following reinstatement of ethanol. However, this increase was temporarily blocked in the BIIE0246 infused rats. No effect of BIIE0246 infusion on ethanol intake was seen in the rats with continuous access to ethanol. Food intake, measured 2 hr post infusion, was also decreased by BIIE0246, suggesting that the effects of BIIE0246 may not be specific to ethanol consumption. This research demonstrates that ICV infusion of a Y2 antagonist blocks an ADE in P rats. These findings are an impetus for research exploring BIIE0246 and its impact on neural modulation of withdrawal symptoms. To the extent that the ADE is an animal model of relapse, it is suggested that Y2 antagonists may be particularly effective as a clinical treatment that possibly alleviates craving in alcohol dependent individuals.

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EXTRAHYPOTHALAMIC CORTICOTROPIN-RELEASING FACTOR MAINTAINS HIGH LEVELS OF ETHANOL CONSUMPTION ASSOCIATED WITH "DRINKING IN THE DARK" PROCEDURES

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Recent evidence suggests that corticotropin-releasing factor (CRF) is involved in maintaining binge-like ethanol consumption observed with drinking-in-the-dark (DID) procedures. The current experiments used DID procedures to further characterize the role of CRF in binge-like ethanol consumption by C57Bl/6J mice. The role of central CRF receptor signaling was assessed in Experiment 1 with intracerebroventricular (i.c.v.) infusion of the non-selective CRF receptor antagonist, α-helical $CRF_{9\text{-}41}$ (0, 1, 5, 10 μg/1ml). The role of the hypothalamic-pituitary-adrenal (HPA) axis was assessed in Experiment 2 by pre-treating mice with 1) the corticosterone synthesis inhibitor, metyrapone (0, 50, 100, 150 mg/kg, intraperitoneal, i.p.) or 2) the glucocorticoid receptor antagonist RU38486 (0, 25, 50 mg/kg, i.p.) and 3) by using radioimmunoassay to determine if binge-like ethanol intake influenced plasma corticosterone levels. The role of the HPA axis was assessed further in Experiment 3 using adrenalectomized (ADX) animals and the CRF1 receptor antagonist, CP-154,526 (CP; 0, 10, 15 mg/kg, i.p.). The possible interaction between CRF and neuropeptide Y (NPY) in binge-like ethanol drinking was assessed in Experiment 4 using NPY knockout mice (NPY-/-) and CP (0, 5, 10, 15 mg/kg, i.p.). Results from Experiment 1 showed that pretreatment with a 1 μg dose of α-helical $CRF_{9\text{-}41}$ significantly attenuated DID consumption relative to vehicle treatment, showing that central CRF receptor signaling modulates binge-like ethanol intake. Experiments 2 and 3 showed that metyrapone non-selectively reduced both ethanol and sucrose consumption, while RU38486 did not alter ethanol drinking. Furthermore, binge-like drinking did not correlate with plasma corticosterone levels, and pretreatment with CP significantly attenuated ethanol intake in both ADX and sham-lesion animals. Results from Experiments 2 and 3 suggest that CRF receptor signaling modulates binge-like ethanol intake independent of the HPA axis. In Experiment 4, CP was more effective in blunting binge-like ethanol drinking in NPY-/- mice relative to NPY+/+ mice, suggesting an interaction between CRF and NPY in the modulation of binge-like drinking. Taken together, the current results indicate the extrahypothalamic CRF, via an interaction with NPY, modulates binge-like ethanol drinking in C57BL/6J mice.

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THE EFFECTS OF STRESS AND NEUROPEPTIDE Y (NPY) SIGNALING ON BINGE-LIKE ETHANOL DRINKING IN C57BL/6J MICE

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Drinking in the dark (DID) procedures has been developed to model binge-like ethanol drinking. This procedure reliably elicits high levels of ethanol consumption and corresponding high blood ethanol levels (BEC) in C57BL/6J mice. Psychological stressors have been shown to increase voluntary ethanol intake in mice, and we have recently found that NPY signaling protects against stress-induced ethanol drinking. The purpose of the present work was to determine if exposure to stress influences binge-like ethanol drinking associated with DID procedures and if NPY plays a modulatory role. To this end, male NPY-/- and NPY+/+ mice (Experiment 1) or Y1 receptor knockout (Y1-/-) and Y1+/+ mice (Experiment 2) maintained on a C57BL/6J background were studied. In Experiment 1, NPY-/- and NPY+/+ mice were subjected to a complex stress procedure for 16-hours/day over 4-days, which involved giving mice an intraperitoneal injection of isotonic saline followed by relocation to a novel environment with white background noise. Mice were then tested for binge-like ethanol consumption using DID procedures 1 and 4 weeks following stress exposure. Results showed that exposure to the complex stress procedure significantly reduced binge-like ethanol intake and corresponding blood ethanol concentrations (BECs) in NPY-/-, but not NPY+/+, mice when tested 1 week after stress procedures. On the other hand, relative to non-stressed animals, exposure to the stress procedure promoted significant reduction of binge-like drinking in both NPY-/- and NPY+/+ mice when tested 4 weeks after stress. In Experiment 2, Y1-/- and Y1+/+ mice were tested using DID procedure before and after a stress procedure involving daily injections of isotonic saline over 5-days. Results showed that there were no genotype differences in pre-stress binge-like ethanol drinking, and that the stress procedure significantly blunted binge-like ethanol intake and associated BECs in Y1-/-, but not Y1+/+, mice. Together, the present results indicate that exposure to psychological stress inhibits binge-like ethanol drinking in C57BL/6J mice, and that NPY, via Y1 receptor signaling, protects against stress-induced reductions of binge-like drinking.

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PRENATAL STRESS INCREASES OPERANT RESPONDING AND ALCOHOL INTAKE DURING ALCOHOL REINFORCEMENT IN ADULT C57BL/6J MICE

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Early environmental insults can alter neurodevelopment, predisposing individuals to neuropsychiatric disorders, including substance abuse. Prenatal exposure to stress has previously been shown to produce disruptions in nervous system development, and an adult behavioral phenotype which is hyperresponsive to stress and psychomotor stimulant drugs. This study examined the impact of prenatal stress (PNS) on the motivation to seek and consume alcohol. Timed pregnant C57Bl/6J dams were subjected to repeated restraint stress from E14 until delivery (1 hr, 3 times daily), while control dams were left undisturbed during gestation. After birth, all groups were left undisturbed until weaning at 21 days into same-sex groups. At 8 weeks of age, 2 male pups were selected from each litter for testing and single housed. All mice were maintained under standard conditions with ad libitum food access. In a two-bottle choice task, mice were allowed continuous access to one bottle of water and one bottle of 15% alcohol in water. No significant differences were found in the two-bottle choice alcohol consumption between PNS and control mice. Next, mice were examined under operant alcohol access conditions. Mice were initially trained to press a lever for oral sucrose reinforcers (20μl volume of 15% w/v sucrose in water) during 15 minute daily sessions. This was followed by a standard sucrose fading procedure, with an unsweetened ethanol solution (20μl of 10% ethanol in water) as the final reinforcer. The PNS and control groups were not significantly different in either operant responding rate or volume consumed during sucrose reinforcement. During ethanol reinforcement, PNS and control groups were significantly different on both measures, with the PNS group showing increased responding on the active lever and increased alcohol consumption compared to controls. These findings indicate PNS increases the motivation for alcohol and that early environmental factors are likely to play a role in the development of alcoholism.

SYMPOSIUM S26-2, POSTER P20-2

NEUROPEPTIDE Y (NPY) Y1 AND CORTICOTROPIN-RELEASING FACTOR (CRF)-1 RECEPTORS MODULATE STRESS-INDUCED INCREASES OF ETHANOL INTAKE IN MICE

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Background and Objectives: Recent work indicates that neuropeptide Y (NPY) receptor agonists, and corticotropin-releasing factor (CRF) receptor antagonists, protect against increases of ethanol intake stemming from ethanol dependence and withdrawal. Here we tested the hypothesis that the NPY Y1 and CRF-1 receptors modulate the effects of stress on ethanol consumption.

Methods: In Experiment 1, mutant mice lacking normal production of the Y1 receptor (Y1-/-) and littermate wild-type mice (Y1+/+) maintained on a C57BL/6J background were trained to drink 15% (v/v) ethanol in daily 2-hour limited access sessions. When a stable baseline was achieved, mice from each genotype were divided into two groups matched for ethanol consumption. Control mice received intraperitoneal (i.p.) injection (20 mg/kg) of pyrazole (1 mmol/kg mixed in 0.9% saline) 30 minutes before placement into a vapor chamber perfused with normal air. Ethanol-exposed mice received i.p. injection of a cocktail of 1.5 g/kg ethanol and pyrazole 30 minutes before placement into a chamber perfused with ethanol vapor. Animals were kept in the chambers for 16 hours/day, and this treatment was repeated over 4 consecutive days. After a 36-hour ethanol withdrawal period, consumption of the 15% ethanol solution was assessed over 5 days. In Experiment 2, BALB/cJ mice were trained to drink 8% (v/v) ethanol 24 hours a day. When a baseline of ethanol consumption was established, animals were exposed to 5 minutes of forced swim stress on each of 5 consecutive days. Thirty minutes before each forced swim session, animals were given an intraperitoneal injection of a 10 mg/kg dose of the CRF-1 receptor antagonist CP-154,526 or an equal volume of vehicle.

Results: Results from Experiment 1 showed that exposure to either the air and ethanol vapor chambers (stressful events) promoted an increase of subsequent ethanol intake and increases of ethanol intake were significantly greater in Y1-/- mice relative to Y1+/+ mice. Results from Experiment 2 revealed that exposure to forced swim stress significantly increased ethanol consumption in the BALB/cJ mice. Importantly, mice pretreated with the CRF-1 receptor antagonist did not exhibit stress-induced increases in ethanol intake, and the CRF-1 receptor antagonist did not influence ethanol drinking in non-stressed mice.

Conclusions: The present results provide evidence that NPY Y1 and CRF-1 receptor signaling modulate increased ethanol consumption stemming from exposure to stressful events.

Impact: Because exposure to stressful life events, including those that promote post-traumatic stress disorder, has been linked to the development of alcohol dependence, compounds aimed at the NPY Y1 and CRF-1 receptors may have therapeutic relevance for the treatment of stress-related alcohol disorders.

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CRF-SAPORIN CONJUATE INJECTED INTO THE BASOLATERAL AMYGDALA PREVENTS SENSITIZATION OF WITHDRAWAL-INDUCED ANXIETY IN A MULTIPLE WITHDRAWAL PARADIGM

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Previous work has demonstrated that repeated withdrawals from chronic ethanol exposure sensitizes withdrawal-induced anxiety-like behavior—a rodent model of "kindling" of negative affect (Overstreet et al. 2002). Likewise, repeated exposure of ICV corticotropin releasing factor (CRF) in rats prior to 5 days of ethanol exposure also sensitizes withdrawal-induced anxiety-like behavior (Breese et al. 2004), suggesting that CRF contributes to the adaptive change responsible for sensitization. In accord with this view, studies have demonstrated that CRF1-receptor antagonists will prevent the cumulative adaptation(s) responsible for the withdrawal-induced anxiety induced by both protocols (Breese et al., 2004; Overstreet et al., 2004). In order to determine if CRF receptor-expressing neurons in the basolateral amygdala (BLA) were involved, CRF conjugated to saporin (CRF-Saporin) (Advanced Targeting Systems, La Jolla, CA), a ribosomal inactivating protein, was microinjected into the BLA of adult Sprague-Dawley rats. A conjugate of a random sequence of amino acids possessing no known affinity for CRF receptors and saporin (Blank-SAP) (Advanced Targeting Systems, La Jolla, CA) was used as a control. All rats received three injections per side of either 0.5 μl of 8.0 ng/1μl Blank-SAP or CRF-SAP that were spaced 0.5 mm apart anterior-posterior. Following surgeries, rats recovered for 3 days before initiating a multiple-withdrawal paradigm of alternating 5 days on, 2 days off 4.5% ethanol liquid diet. Six hours into the third withdrawal, rats were tested for locomotor activity and social interaction, an index of anxiety-like behavior in the rat. The targeted ablation of CRF receptor-expressing cells within the BLA restored social interaction to control levels suggesting a prominent role of these cells in the complex neural circuitry involved in withdrawal-induced anxiety-like behaviors. Future analysis of brain tissue is underway to determine the identity and extent of the ablated cells. AA-11605 & AA014949.

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NPY Y1 RECEPTOR KNOCKOUT MICE SHOW INCREASE SENSITIVITY TO STRESS-INDUCED INCREASES OF ETHANOL INTAKE AND WITHDRAWAL-INDUCED ANXIETY-LIKE BEHAVIOR

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Neuropeptide Y (NPY) been implicated in the modulation of anxiety-like behavior, stress responses, and neurobiological responses to ethanol. Recent work indicates that NPY protects against increases of ethanol intake and anxiety-like behavior stemming from ethanol dependence and withdrawal. Here we tested the hypothesis that the NPY Y1 receptor modulates the effects of ethanol exposure and withdrawal on subsequent ethanol consumption and anxiety-like behavior using mutant mice lacking normal production of the Y1 receptor (Y1-/-). Using a modified sucrose fading procedure, male and female Y1-/- and littermate Y1+/+ mice (C57BL/6J background) were trained to drink 15% (v/v) ethanol in daily 2-hour limited access sessions. When a stable baseline was achieved, mice from each genotype were divided into two groups matched for ethanol consumption. Control mice received intraperitoneal (i.p.) injection (20 mg/kg) of pyrazole (1 mmol/kg mixed in 0.9% saline) 30-minutes before placement into a vapor chamber perfused with normal air. Ethanol exposed mice received i.p. injection of a cocktail of 1.5 g/kg ethanol and pyrazole 30-minutes before placement into a chamber perfused with ethanol vapor. Animals were kept in the chambers for 16-hours/day and this treatment was repeated over 4 consecutive days. After a 36-hour ethanol withdrawal period, consumption of the 15% ethanol solution was assessed over 5-days. Vapor exposure and limited access ethanol intake as described above was repeated over 2 additional cycles. At then end of the experiment, mice were tested in a novel open-field environment to assess anxiety-like behavior. The results showed that exposure to either the air and ethanol vapor chambers (stressful events) promoted an increase of subsequent ethanol intake and increases of ethanol intake were significantly greater in Y1-/- mice relative to Y1+/+ mice. Furthermore, repeated exposure to ethanol vapor and withdrawal increased anxiety-like behavior revealed by reduced locomotor activity in the center portion of the open-field apparatus. Importantly, Y1-/- mice showed significantly greater sensitivity to withdrawal-induced anxiety-like behavior relative to Y1+/+ mice. These results suggest that the Y1 receptor protects against stress-induced increases of ethanol consumption and anxiety-like behavior stemming from ethanol exposure and withdrawal. (Supported by NIH grants AA013573, AA015148, and the Department of Defense grant W81XWH-06-1-0158).

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SITE-SELECTIVE EFFECTS OF CRF ON ETHANOL WITHDRAWAL INDUCED ANXIETY: PHARMACOLOGICAL REGULATION

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Previous work showed that anxiety-like behavior was increased in rats repeatedly injected with CRF prior to a 5-day exposure to ethanol. The present studies sought to determine which brain regions mediate these effects, and which pharmacological agents modulate the effects. In the first experiment rats were cannulated in either the central amygdala (CeA), the dorsal raphe nucleus (DRN), or the paraventrricular nucleus (PVN) of the hypothalamus. The rats were placed on control liquid diet and then injected with CRF (0.5 μg in 0.5 μl) twice at 5-day intervals. Then the rats were exposed to an ethanol-containing diet for five days and withdrawn. Social interaction and locomotor activity were assessed 5 hr after the ethanol was withdrawn. Rats that were injected with CRF into the CeA and DRN exhibited anxiety-like behavior, while rats injected into the PVN exhibited normal social behavior. Subsequently, rats with cannulae in the CeA and DRN were pretreated with buspirone, a 5-HT1A partial agonist, flumazenil, a benzodiazepine antagonist, and SSR125543, a CRF1 receptor antagonist, prior to the CRF injections. All drugs were effective in blocking the anxiogenic effects of CRF administered into the DRN. Only flumazenil and SSR125543 were effective when CRF was administered in the CeA. These findings are consistent with the view that extrahypothalamic CRF systems mediate its effects on emotional behavior. Pathways in the CeA and DRN are very important, but can be modulated by a benzodiazepine antagonist as well as a CRF1 receptor antagonist.

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A HISTORY OF ETHANOL-DEPENDENCE PRODUCES AN INCREASED SENSITIVITY TO THE ANTIRELAPSE ACTION OF NOCICEPTIN IN AN ANIMAL MODEL OF STRESS-INDUCED RELAPSE

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Stress is a major risk factor that contributes to relapse in abstinent alcohol-dependent individuals. Amelioration of this exacerbated stress is, therefore, an important goal when considering pharmacotherapies targeting prevention of relapse to drug use. The anxiolytic-like actions of nociceptin/orphanin FQ (N/OFQ), a N/OFQ receptor agonist, have been described in the literature. However, the effects of this agent have not been compared in ethanol-dependent vs. non-dependent animals—an important consideration in the clinic. The present study examined for any differential effects of N/OFQ, in ameliorating electric footshock stress-induced reinstatement (a valid animal model of relapse evoked by stress) of ethanol-seeking in ethanol-dependent vs. non-dependent rats. Following operant ethanol (10% w/v) self-administration training, rats were made dependent by intragastric intubation of 10 g/kg of ethanol per day (administered for 6 consecutive days in a dietary liquid vehicle 4 times a day). Peak blood alcohol levels associated with this treatment reached 200-250 mg% and severe somatic withdrawal symptoms were observed on day 6 (12 h following the last ethanol administration). One week following termination of ethanol intoxication, both groups of rats were allowed to self-administer 10% ethanol until stable responding, after which they underwent at least 14 extinction sessions. Subsequent exposure to a 15 min intermittent footshock (0.5 mA, 0.5 s duration, VI 40 s) elicited robust reinstatement of responding at the previously alcohol-paired lever. Pretreatment with N/OFQ (0.5, 1.0, 2.0 μg, ICV) reduced stress-induced reinstatement in the non-dependent group at the 2 μg dose, as previously reported, while in the ethanol-dependent group, N/OFQ inhibited stress-induced reinstatement at both 1 and 2 μg. These observations suggest that a history of ethanol dependence is associated with an increased sensitivity to N/OFQ in inhibiting stress-induced reinstatement. Furthermore, this differential effect of N/OFQ in ethanol-dependent vs. non-dependent animals suggests that neuroadaptive changes related to the N/OFQ system result from a history of ethanol dependence. (Supported by NIH/NIAAA grant AA 014351).

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ETHANOL-DEPENDENT RATS SHOW DIFFERENTIAL SENSITIVITIES TO THE ANTI-RELAPSE EFFECTS OF MGLU2/3 AND MGLU5 RECEPTOR LIGANDS

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Heightened stress is a major contributing factor to the high relapse rates in abstinent alcoholics. There is growing evidence to support the role of metabotropic glutamate (mGlu) receptors in ethanol drinking and ethanol-seeking behavior in rats. Moreover, neuroadaptation in the function of these receptors that occurs during chronic exposure to ethanol may play a pivotal role in ethanol dependence and the propensity to relapse. Both the mGlu2/3 receptor agonist, LY379268, and mGlu5 receptor antagonist, MTEP, have been shown to have anxiolytic actions. Hence, the present study examined for any differential effects of LY379268 and MTEP in ameliorating stress-induced reinstatement of ethanol-seeking (i.e., an animal model of relapse evoked by stress) in ethanol-dependent vs. non-dependent rats. Following operant ethanol (10% w/v) self-administration training, rats were made dependent by intragastric intubation of ethanol, administered for 6 consecutive days in a dietary liquid vehicle 4 times a day (for a total of 10 g/kg/day). After 5 days, peak blood alcohol levels associated with this treatment regimen reached 200-250 mg% in the ethanol-treated group, and on day 6 (12 h following the last ethanol administration), severe somatic withdrawal symptoms were observed. One week following termination of ethanol intoxication, both groups of rats were allowed to self-administer 10% ethanol until stable responding, after which they underwent at least 14 extinction sessions. Subsequent exposure to 15 min intermittent footshock (0.5 mA, 0.5 s duration, 10-70 s VI) elicited robust reinstatement of responding at the previously ethanol-paired lever. Pretreatment with LY379268 (0.3, 1.0, 3.0 mg/kg; SC) or MTEP (0.3, 1.0, 3.0 mg/kg; IP) dose-dependently reduced footshock stress-induced reinstatement of ethanol-seeking in ethanol-dependent and non-dependent rats. Compared to non-dependent animals, ethanol-dependent rats were more sensitive to the effects of LY379268, and conversely, non-dependent rats were more sensitive to the effects of MTEP. These observations suggest that a history of ethanol dependence may differentially alter the sensitivity and/or number of mGlu2/3 and mGlu5 receptors. (Supported by NIAAA AA10531 [FW])

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THE EFFECTS OF STRESS ON ETHANOL CONSUMPTION IN BALB/CJ, DBA/2J, AND C57BL/6J MICE

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Recent evidence suggests that ethanol consumption, following exposure to stressful events, increases among initially low ethanol consuming animals but is not changed or decreases among initially high ethanol consuming rodents. The current experiment was conducted to further assess the relationship between initial ethanol intake and the effects of stress exposure on ethanol consumption in DBA2J, BALB/cJ, and C57BL/6J mice, strains that drink low, moderate, and high amounts of ethanol, respectively. After approximately one week of habituation to the housing facilities, all animals were given continuous access to a 2% (v/v) ethanol solution and tap water using 24-hour two-bottle preference procedures. The concentration of ethanol solution was increased every 4-days until reaching a final concentration of 8% ethanol. Once baseline consumption of the 8% solution had stabilized, animals of each strain were then divided into Stress and No Stress groups equated for baseline ethanol consumption. All animals of the Stress groups were exposed to 6 consecutive days of stress exposure, which consisted of 3-days of exposure to forced swim procedures (5-minutes/day) and 3-days of exposure to footshock procedure (5-minutes/day; 5 mA, 5 shocks/5 minutes). Exposure to forced swim stress or shock were randomly presented between days. Animals of the No Stress groups were handled as a control for forced swim stress, and were exposed to shock chambers as a control for footshock stress. Ethanol consumption was monitored every 2 days for four weeks following the stress exposure period. Food, water, and ethanol were available ad libitum for the duration of the experiment. Results revealed a significant negative correlation between initial ethanol intake and the subsequent effects of stress on ethanol drinking. Thus, while mice with low or moderate (DBA/2J and BALB/cJ) ethanol consumption showed stress-induced increases of ethanol consumption, high ethanol consuming C57BL/6J mice showed stress-induced reductions of ethanol consumption. The results of this experiment indicate that initial ethanol preference may predict the effects of stress on later ethanol consumption. The effects of stress on ethanol consumption by other inbred strains of mice is currently under investigation. (This work was supported by NIH grants AA013573, and AA015148, and Department of Defense grant W81XWH-06-1-1058).

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EFFECTS OF SOCIAL DEFEAT ON ETHANOL DRINKING AND ANXIETY MEASURES

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Human data strongly suggests that stressful life situations increase alcohol abuse and relapse drinking. However, findings in rodent models are inconsistent: increases, decreases or no change in ethanol drinking patterns following a variety of stressors have been reported. Moreover, it has not always been clear if the stress paradigm produces a physiological response that is relevant at the time of ethanol self-administration. Here, we have designed experiments to test the effects of repeated social defeat on ethanol drinking in mice and whether neurochemical measures of HPA axis activation and behavioral measures of anxiety are reflected in these changes.

C57BL/6 mice were tested for basal anxiety-like behavior in the light-dark transition model prior to the initiation of drinking studies. Two groups were given voluntary access to 10% (w/v) ethanol and tap water in a 2 bottle choice paradigm while two groups were given access to only water. Following establishment of stable drinking patterns, half the mice were exposed to brief social defeat by an aggressive male or remained in their home cage for 5 consecutive days. Ethanol was continually available in these studies. Separate groups of mice were tested in the light-dark box following baseline ethanol drinking and social defeat. Corticosterone levels were measured 24 hours following the last social defeat as a measure for HPA axis activation. These measures will be used to explore how social stress modifies ethanol drinking.

We have previously shown that social defeat stress may have bidirectional effects on ethanol drinking in C57BL/6 mice. Mice with a predilection for low ethanol preference tend to increase drinking following social stress while high preference mice tend to decrease drinking. We have extended these studies in another inbred strain, 129SvJ, which consumes moderate amounts of ethanol. Interestingly, 129SvJ mice with baseline drinking intake less than 4g/kg in 24 hours increase drinking following social defeat. Mice with greater than 4 g/kg baseline intake either decreased or did not change their intake following social defeat. Together, investigation of social stress modifications on individual variation of ethanol drinking coupled with behavioral and neurochemical measures will help elucidate role of stress in alcohol abuse. Supported by NIAAA Grant R01 AA14717 to MFM and AA16454 to JTW.

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THE ROLE OF STRESS AND CYTOKINES IN THE SENSITIZATION OF ANXIETY-LIKE BEHAVIOR IN ADOLESCENTS

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Previous research has illustrated that repeated ethanol withdrawals sensitizes anxiety-like behavior ("anxiety") in adolescent and adult rats. Additionally we have demonstrated that stress, lipopolysaccaride (LPS), and cytokines (TNFα, MCP-1, & IL-1β) are able to substitute for early withdrawals and produce this sensitized anxiety following only a single 5-day cycle of ethanol in adults. The present experiments were performed to determine the effects of stress and cytokines on the sensitization of this anxiety in adolescents. Adolescent rats were tested in the stress/withdrawal paradigm, in which stress (60 min restraint stress) is substituted for early withdrawals followed by a 5-day cycle of 2.5% ethanol diet. Results showed that stress substituted for early ethanol withdrawals and produce a sensitization of anxiety during withdrawal from the ethanol exposure. In addition, TNFα was given intraventricularally (icv) at weekly intervals in place of early withdrawals and demonstrated a sensitization of anxiety in adolescents. Further, experiments in adolescents were performed to determine the acute effects of restraint stress on both anxiety and TNFα. In adults, it has been demonstrated that 60 minutes of restraint stress is sufficient to acutely induce anxiety and increase TNFα in brain. Therefore, it was tested whether various amounts of restraint stress (45, 60, 90, 120 minutes) were able to acutely induce anxiety (measured by social interaction 30 min following stress) and increase TNFα (from whole brains collected 5 hr after stress) in adolescents. Results illustrated that in adolescents any amount of restraint stress tested were insufficient to acutely produce anxiety. This result is in contrast to adults, where 60 minutes of restraint stress is sufficient to produce anxiety. However, when TNFα was measured in adolescents, it was shown to be increased in groups that received 45 and 90 minutes of restraint stress compared to non-stressed controls. These data have illustrated that both stress and cytokines (TNFα) are able to substitute for early withdrawals to sensitize anxiety in adolescents. Additionally, it was highlighted that the acute responses to stress during adolescents may be different from those of adults. Future studies will explore possible explanations for this age-dependent difference. Supported by AA-16704, AA-11605, AA-14949.

Program#/Poster#: 270.10/X24

Title: Amygdalar neuropeptide Y (NPY) signaling modulates stress-induced reductions of food intake in Balb/cJ mice

Location: San Diego Convention Center: Halls B-H

Presentation Start/End Time: Sunday, Nov 04, 2007, 2:00 PM - 3:00 PM

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The existing literature suggests that NPY signaling in the amygdala modulates anxiety-like behaviors and ethanol consumption in rodents, but does not modulate food intake. On the other hand, NPY signaling within the hypothalamus controls food intake but does not influence anxiety-like behavior.
Based on these observations, the current study tested the hypothesis that attenuation of NPY signaling within the amygdala would increase anxiety-like behavior and augment stress-induced increases of ethanol consumption while at the same time have no effect of feeding behavior. To address this hypothesis, male Balb/cJ were given bilateral injection (48 ng/5-min/side) into the central nucleus of the amygdala (CeA) of NPY conjugated to the neurotoxin saporin (NPY-SAP) or saporin alone (Blank-SAP). NPY-SAP is a ribosome inactivating neurotoxin that targets and kills cells expressing NPY receptors. After recovery, mice were first tested for anxiety-like behavior using the zero maze test. They were then given access to 8% (v/v) ethanol versus water in a two-bottle test. After ethanol intake stabilized, half the NPY-SAP and Blank-SAP mice were subjected to a 5-min forced swim stress sessions, once a day over 5-days. Ethanol, water and food consumption were measured for 4-weeks following the forced swim procedures. At the end of the experiment, ethanol was removed for two-weeks and all mice were given a 24-hour open-field locomotor activity test. The results showed that mice treated with NPY-SAP in the CeA spent significantly less time in the open portion of the zero maze reflecting elevated anxiety-like behavior. Contrary to predictions, neither neurotoxin treatment nor stress condition altered ethanol intake. Interestingly, NPY-SAP treated mice that experienced forced swim stress consumed significantly less food than non-stressed NPY-SAP treated mice and stress and non-stressed mice treated with the Blank-SAP. Reduced feeding by NPY-SAP stressed mice was not associated with reduced body weight, suggesting possible alterations of energy metabolism. Further, reduced feeding was not attributable to reductions of activity. This study provides novel evidence that amygdalar NPY signaling modulates feeding/energy balance in mice with a history of stress exposure.

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Title: The CRF-1 receptor antagonist, CP-154,526 (butyl-[2,5-dimethyl-7-(2,4,6-trimethylphenyl)-7H-pyrrolo[2,3-d]pyrimidin-4-yl]-ethylamine), attenuates stress-induced increases in ethanol consumption in BALB/CJ, but not C57BL/6N, mice

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Corticotrophin-releasing factor (CRF) signaling modulates stress and ethanol consumption, and may modulate observed increases in ethanol consumption following exposure to stressful events. The current experiment was conducted to further characterize the role of CRF-1 receptor signaling in stress-induced increases of ethanol consumption in BALB/cJ and C57BL/6N mice. Male BALB/cJ and C57BL/6N mice were given continuous access to 8% ethanol and water for the duration of the experiment. When a baseline of ethanol consumption was established, animals were exposed to five minutes of forced swim stress on each of five consecutive days. Thirty minutes before each forced swim session, animals were given an intraperitoneal injection of a 10 mg/kg dose of CP-154,526, a selective CRF-1 receptor antagonist, or an equal volume of vehicle. Results revealed that exposure to forced swim stress significantly increased ethanol consumption in the BALB/cJ, but not in the C57BL/6N, mice. Stress-induced increases in ethanol consumption occurred approximately two weeks after the first stressor and were not associated with increased food or water consumption. As an additional consummatory control, we are currently investigating the effects of stress on sucrose consumption. Importantly, BALB/cJ mice pretreated with the CRF-1 receptor antagonist did not exhibit stress-induced increases in ethanol intake, and the CRF-1 receptor antagonist did not influence ethanol drinking in non-stressed mice. The present results provide evidence that CRF-1 receptor signaling modulates increased ethanol consumption stemming from repeated exposure to a stressful event in BALB/cJ mice.

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Title: Amygdalar transduction by a rAAV vector causing constitutive secretion of NPY blocks the alcohol deprivation effect and anxiety-like behavior in Alcohol Preferring P rats

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The Indiana Alcohol Preferring P rats were selectively bred to consume large amounts of ethanol. Previous work showed that when compared to Alcohol Non-Preferring NP rats, P rats have significantly lower levels of neuropeptide Y (NPY) in the amygdala and ventricular infusion of exogenous NPY significantly reduces ethanol drinking in P rats. Here, we determined if overexpression of NPY in the central nucleus of the amygdala (CeA) of P rats would protect against increased ethanol drinking associated with the alcohol deprivation effect (ADE) and/or elevated anxiety-like behavior during deprivation periods. To this end, P rats were given bilateral infusion into the CeA of a recombinant adeno-associated viral (rAAV) vector contained the fibronectin secretory signal sequence followed by the coding sequence for NPY (rAAV-FIB-NPY), a construct that causes constitutive secretion of vector-derived NPY from transduced cells. Control rats were injected with an equal volume of saline. In separate studies, vectors was injected into the amygdala 10-days before exposure to ethanol or after ethanol drinking was established. Following baseline two-bottle (10% ethanol versus water) ethanol consumption, the P rats were placed on an ADE protocol where ethanol access was available in cycles of 5-days followed by 2-days of deprivation. Results showed that P rats with low ethanol drinking (1.5 - 3.5 g/kg/day) exhibited an ADE that was not significantly altered by the rAAV-FIB-NPY vector. However, saline-treated P rats exhibited anxiety-like responses in a social interaction test during deprivation periods that were blocked in rats treated with the rAAV-FIB-NPY vector. Similarly, no effect of the vector on ADE ethanol drinking was observed in P rats after moderate levels of ethanol drinking (2.5 - 5 g/kg/day) had been established. In contrast, in P rats that had high levels of ethanol intake (4 - 8 g/kg/day), the rAAV-FIB-NPY vector eliminated ADE-induced increases of ethanol drinking (0.29 g/kg/day increase) relative to P rats treated with saline (1.42 g/kg/day increase). These results show that chronic expression and constitutive release of NPY from cells in the amygdala can exert relevant antagonistic effects in models of ethanol intake and deprivation-induced anxiety. These effects may depend on the basal level of ethanol intake and suggest that efficacy of NPY activity in these models may best be seen in animals that are experienced with high levels of voluntary ethanol intake. (Supported by NIH grants AA013573, AA015148, AA011605, NS35633, and Department of Defense grant PR054214).

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THE CRF-1 RECEPTOR ANTAGONIST, CP-154,526, ATTENUATES STRESS-INDUCED INCREASES IN ETHANOL CONSUMPTION IN BALB/CJ, BUT NOT C57BL/6N, MICE

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Corticotrophin-releasing factor (CRF) signaling modulates stress and ethanol consumption, and may modulate observed increases in ethanol consumption following exposure to stressful events. The current experiment was conducted to further characterize the role of CRF-1 receptor signaling in stress-induced increases of ethanol consumption in BALB/cJ and C57BL/6N mice. Male BALB/cJ and C57BL/6N mice were given continuous access to 8% ethanol and water for the duration of the experiment. When a baseline of ethanol consumption was established, animals were exposed to five minutes of forced swim stress on each of five consecutive days. Thirty minutes before each forced swim session, animals were given an intraperitoneal injection of a 10 mg/kg dose of CP-154,526 or an equal volume of vehicle. Results revealed that exposure to forced swim stress significantly increased ethanol consumption in the BALB/cJ, but not in the C57BL/6N, mice. Stress-induced increases in ethanol consumption occurred approximately two weeks after the first stressor and were not associated with increased water consumption. Importantly, BALB/cJ mice pretreated with the CRF-1 receptor antagonist did not exhibit stress-induced increases in ethanol intake, and the CRF-1 receptor antagonist did not influence ethanol drinking in non-stressed mice. The present results provide evidence that CRF-1 receptor signaling modulates increased ethanol consumption stemming from repeated exposure to a stressful event in BALB/cJ mice. (Supported by NIH grants AA015148 and AA013573, and the Department of Defense grant PR054214).

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ETHANOL INTAKE IN WISTAR KYOTO RATS DURING CHRONIC MILD STRESS

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Certain psychological disorders, such as major depressive disorder (MDD) and alcoholism, have a high rate of comorbidity. Prevalence rates for comorbid alcohol use are about one quarter of patients diagnosed with MDD. These disorders have similar risk factors, including stressful life events, psychological trauma and genetic vulnerability. A majority of patients reported that the onset of depression took place prior to alcohol use. We sought to determine the effect of stress on ethanol intake in a model of primary depression. Adolescent Wistar and Wistar Kyoto (WKY) rats were used because they have been proposed as an animal model of depression. Rats were randomly assigned to one of five groups: control (no ethanol/no stress), ethanol (no stress), stress (no ethanol), stress/ethanol, or stress/yoked ethanol. Rats in the stress groups were exposed to a chronic mild stress (CMS) paradigm for 28 days. Rats in the ethanol groups were given access to two bottles: 5% (v/v) ethanol and water starting on day 8. Drinking volume was measured and bottles were switched daily. Rats in the stress/yoked ethanol group were given the same amount of ethanol (g/kg/day) as a weight-matched ethanol group rats of the same strain. Locomotor Activity was assessed at 2 time points: prior to the start of week one and after the end of week four. WIS rats gained less weight (34%) compared to WKY rats (38%). In addition, WKY rats exhibited a decreased food intake (–4.7%) vs. WIS rats (.52%), with the stress groups having the greatest increase in food intake (3.68%). Results also showed that WKY ethanol rats had increased ethanol intake (2.6 g/kg/day) and preference (42%) compared to WIS ethanol rats (1.86 g/kg/day; 31%). Preliminary results indicated that WKY stress/ethanol rats had decreased ethanol intake by week 4 (1.73 g/kg/day) compared to the WKY ethanol rats (2.88 g/kg/day). These results will be compared with previous data and the self-medication hypothesis that purports that alcohol is used to alleviate stress (Khantzian 1995). Supported by the NIAAA (Intramural Research Program AA 11034 & AA07574, AA07611).

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BRIEF REPEATED SOCIAL DEFEAT ALTERS ETHANOL DRINKING PATTERNS IN C57BL/6 MICE

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Humans have long reported increased drinking following stressful periods. Stress has been shown to increase ethanol drinking in a number of behavioral models, although not in all studies. Repeated social defeat (RSD) is an ethologically relevant model of stress for rodents representing situations the animal would normally encounter in its natural environment and for which it may have developed evolutionary defenses. RSD has been shown to alter neurochemical measures of anxiety, increase cardiac and adrenocortical responsiveness as well as increase ethanol drinking in low preference mice. This model, however, has also been shown to decrease or have no effect on ethanol drinking in rodents. Despite a number of reports looking at the effects of social stress on ethanol drinking, direct study of the molecular networks involved in this behavior has not been investigated. We are interested in determining the molecular pathways activated by social stress which contribute to drinking behaviors. The current experiments were designed to test the effects of RSD on ethanol drinking. Male C57BL/6 mice were given voluntary access to 10% (w/v) ethanol and tap water in a 2 bottle choice paradigm. Following stable drinking patterns, mice were exposed to brief social defeat by an aggressive male conspecific or briefly exposed to a clean cage as control for 5 consecutive days. In one study, mice had no access to ethanol during RSD but access was resumed for two weeks following defeat. In this study, ethanol drinking levels decreased in the defeated mice at 2 days up to 2 weeks upon ethanol reinstatement. However, mice with initial low drinking levels increased their ethanol intake following social defeat. These results suggest that the level of ethanol drinking prior to social stress may be a key determinant for drinking responses following stress. Additional experiments investigating the effect of RSD with concurrent ethanol access will be presented. Brain regions from individual mice subjected to social defeat and voluntary ethanol drinking will then be analyzed using Affymetrix microarrays to identify gene networks contributing to social stress modulation of ethanol drinking. Supported by NIAAA Grant R01 AA14717 to MFM.

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EFFECT OF CHRONIC ETHANOL ON 5HT1A AND SEROTONIN TRANSPORTER DENSITIES IN THE HIPPOCAMPUS AND RAPHE OF MOTHER-REARED VS PEER-REARED MACAQUES

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Early childhood stress may fundamentally alter brain and neuroendocrine development, and evidence suggests these alterations may underlie an increase in the risk for excessive ethanol self-administration later in life. The hippocampus and raphe nuclei are two of many brain regions that have been shown to be involved not only in the modulation of the serotonergic component of the stress response, but are also part of the brain reward circuitry involved in the development of excessive drinking behavior. Eight male rhesus macaques [mother-reared (n=4) or peer-reared (n=4)] were induced to self-administer ethanol using a schedule-induced polydipsia procedure. Following induction, monkeys self-administered ethanol or concurrently available water during 22 hr sessions in their home cage over a period of 12 months. At necropsy, brains were blocked, flash-frozen, and subsequently processed for in vitro receptor autoradiography using the 5HT1-A receptor antagonist [3H]MPPF and the serotonin transporter ligand [3H] Citalopram. Non-specific binding was determined using WAY-10065 or fluoxetine, respectively. Images were analyzed using AIS software and regions of interest were confirmed using overlaid nissl stained images of the same sections. Within the hippocampus, the overall density of serotonin 5HT1-A receptors were not different between rearing conditions; however, significant differences were seen in 5HT1-A densities in the pyramidal cell layer of CA4 (p=.05, increased in NR group) and in the stratum lacunosum moleculare of CA3 (p=.01, decreased in NR group). There were no overall or layer specific differences in serotonin transporter density between the two rearing conditions. In addition, there were no significant differences between rearing conditions in 5HT1-A or SERT binding density in the raphe of these animals. In conclusion, chronic ethanol self-administration may have resulted in significant CA field and layer specific changes in 5HT1-A receptor densities in the hippocampus, but not transporter densities in the hippocampus or raphe of PR vs MR young adult macaques. This study was supported by U01-AA-014106.

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EFFECTS OF IMMUNONEUTRALIZATION OF BETA-ENDORPHIN IN THE NUCLEUS ACCUMBENS ON INTRAVENOUS ETHANOL SELF-ADMINISTRATION IN RATS

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The endogenous opioid system has long been implicated in the reinforcing effects of ethanol, as evidenced by the ability of the opioid antagonist naltrexone to reduce ethanol consumption and relapse. Most studies examining the role of individual opioid peptides in ethanol reinforcement have been conducted utilizing mice carrying a targeted deletion of the gene encoding one of the opioid peptide precursors (i.e., preproenkephalin, pro-opiomelanocortin, etc.). A more direct approach to studying the role of opioid peptides in the reinforcing effects of ethanol is the use of immunoneutralization techniques, whereby an antibody directed against a specific opioid peptide is delivered directly into a particular brain region, thereby binding to extracellular peptides and preventing them from interacting with their cognate receptor(s). The goal of this study was to examine the effect of immunoneutralization of beta-endorphin in the nucleus accumbens (Nac) on the reinforcing effects of ethanol. Male Wistar rats were implanted with indwelling jugular vein catheters, as well as intracranial microinjection guide cannula aimed at the Nac (shell and core regions), and trained to self-administer ethanol intravenously (1% v/v solution) on an FR1 schedule of reinforcement. Self-administration sessions (1 hr in length) were conducted daily until responses rates on the active lever stabilized. Microinjections were then performed immediately prior to subsequent 1 hr self-administration sessions. Infusion of an anti-beta-endorphin antibody (1 ug/ul total protein content, 0.5 ul/side delivered) into the NAc shell resulted in a 50% reduction in ethanol-reinforced lever presses as compared to artificial CSF vehicle injections. Infusion of a control antibody (anti-actin) into this region had no effect. Experiments examining the effects of beta-endorphin immunoneutralization in the Nac core subregion are currently being conducted. In accord with previous reports showing that ethanol stimulates endorphin release in the Nac, our data suggest that endorphinergic neurotransmission in this region mediates the reinforcing effects of ethanol. This research was supported by a grant from the Alcoholic Beverage Medical Research Foundation and by training grant T32 AA007474 from NIAAA.

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EFFECT OF SEX AND β-ENDORPHIN ON ETOH SELF-ADMINISTRATION IN MICE: OLD QUESTION, NEW FINDINGS

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We previously reported differences in EtOH oral self-administration in transgenice mice with varying levels of β-endorphin (Grisel et al., 1999). In that study we found marginal effects of genotype, with heterozygotes (50% of normal β-endorphin) drinking the most EtOH. However, in this study, only concentrations of 7 and 10% EtOH were evaluated. In order to further delineate the effects of β-endorphin on EtOH reinforcement we repeated this study using a larger range of EtOH concentrations. Male and female, adult, naive, β-endorphin deficient mutant mice (KO; fully backcrossed onto C57BL/6J), heterozygotes (HT) and wildtype controls (B6; C57BL/6J), all bred in-house from progenitors obtained from the Jackson Laboratories (Bar Harbor, ME) were single housed in Plexiglas cages with corn cob bedding and ad lib access to food and water. A two-bottle free choice EtOH oral self-administration paradigm was administered as subjects received 24-hr access to increasing concentrations of EtOH (0%, 3%, 6%, 12%, and 15%) each given for 8 days. Preference and consumption were determined each day and bottles were switched every other day. Overall, homozygous mutant mice (KO) showed decreased preference for EtOH but did not self-administer significantly less (in terms of g/kg) than HT or B6 mice. However, this effect was sex dependent with male KO mice displaying the lowest preference. In fact, when the sexes were analyzed separately, there was a tendency ($p = .06$) for KO males to consume less EtOH across all doses in a repeated measure ANOVA. Females drinking patterns (either preference or dosage) did not differ according to β-endorphin levels. These data support the hypothesis that β-endorphin may contribute to the reinforcing effects of EtOH in a sex-dependent manner.
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POTENTIAL ROLE OF THE NEUROKININ-1 (NK1) RECEPTOR IN ALCOHOL CONSUMPTION

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There are few effective medications currently available for the treatment of alcoholism despite ethanol's devastating impact on individuals addicted to ethanol as well as on the society. Furthermore, the medications currently approved for alcoholism are limited by severe side effects and compliance issues. There is a great need for the development of more effective medications for the treatment of alcoholism. The neurokinin-1 (NK1) receptor is the preferred receptor for substance P, which previously has been studied for its role in depression and anxiety. Recently, the NK1 receptor has been shown to have a role in addiction. In the present study we examined a possible role for the NK1 receptor antagonist LY303870 in treatment of alcoholism. Thus, the consumption of ethanol after LY303870 treatment (1, 10 or 20 mg/kg) was measured in two different paradigms. 1) Continuous-access-two-bottle-choice, when the rats were given unlimited access to ethanol and water. Consumption of ethanol (g/kg) and water (ml) was recorded 16 and 24 after administration of the drug. 2) Operant self-administration of ethanol, when consumption (g/kg) and the number of lever presses for ethanol was recorded during a 30-minute session. Each rat was given four consecutive i.p. injections of vehicle, 1, 10 or 20 mg/kg of LY303870 respectively in random order with 7 days a part. Thus, each rat served as its own control. LY303870 was given as an acute dose 30 minutes before ethanol and water bottles were presented or before the rats were placed in the operant self-administration chambers. We showed that a single injection of LY303870 reduced ethanol intake using both the continuous-access-two-bottle choice and the operant ethanol self-administration paradigm. Water consumption was not affected in the two-bottle-choice paradigm. Furthermore, LY303870 did not affect the locomotor activity in an open filed chamber compared to controls. This rule out the possibility that the decreased response in the self-administration chamber after LY303870 treatment would be due to decreased mobility. In conclusion, the present study confirms that the NK1 receptor may be involved in addiction and that the NK1 receptor antagonist LY303870 could be a candidate for a novel treatment of alcoholism

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ASSESSMENT OF ETHANOL CONSUMPTION FOLLOWING SITE-DIRECTED INFUSION OF A NEUROPEPTIDE Y-SAPORIN NEUROTOXIN IN C57BL/6J MICE

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Relative to low ethanol drinking DBA/2J mice, high ethanol drinking C57BL/6J mice have low levels of neuropeptide Y (NPY) in the nucleus accumbens (NAc) and the central and basolateral nuclei of the amygdala. We have previously shown that increasing central NPY levels in C57BL/6J mice via a recombinant adeno-associated virus leads to brain region-specific alterations of ethanol consumption as well as sensitivity to the locomotor stimulant effects of ethanol. NPY conjugated to Saporin (NPY-SAP) is a ribosomal inactivating toxin which selectively targets and kills cells expressing NPY receptors. In the present study, we utilized NPY-SAP to investigate the effect of reducing NPY signaling in specific brain regions on neurobiological responses to ethanol in C57BL/6J mice. In two separate experiments, we infused either NPY-SAP or Blank-Saporin (B-SAP) at 48 ng/0.5 µ>l bilaterally into the core of the NAc or the amygdala of male and female C57BL/6J mice. Results indicated that after an extensive history of ethanol exposure, mice with blunted NPY signaling in the NAc (the NPY-SAP group) consumed significantly less ethanol during a 4-hour limited access paradigm over 4-days of testing relative to control B-SAP treated mice. Importantly, treatment groups did not differ in water consumption, ingestion of a sucrose solution, or food intake. Interestingly, the effects of the NPY-SAP neurotoxin were brain region specific as we did not observe any effects on ethanol drinking following amygdalar infusion of NPY-SAP. However, amygdalar infusion of NPY-SAP increased sensitivity to the sedative effects of a 1.5 g/kg dose ethanol revealed by an open-field locomotor activity test. The present results indicate that decreasing NPY signaling with NPY-SAP alters neurobiological responses to ethanol in a brain region-specific manner. (Supported by NIH grants AA013573, AA015148, AA016716 and the Department of Defense grant PR054214).